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13. ABSTRACT (Maximum 200 Words) <p>The objective of the present research is to determine whether there is a coherent body of evidence implicating oxidative damage in the pathogenesis of Parkinson's Disease and the MPTP model of Parkinsonism. In the past year, we have developed a novel column switching assay for measurement of oxidative damage to DNA in human body fluids. We have applied to this plasma samples of Parkinson's Disease patients. We have also developed a novel technique for directly looking for mitochondrial DNA mutations in isolated neurons of the substantia nigra of human postmortem brain. We have validated this methodology. We have found a relatively high mutation rate and control samples and intend to apply this to Parkinson's Disease. We have continued our studies to determine whether oxidative damage plays a critical role in MPTP toxicity. We have found that mice which are deficient in manganese superoxide dismutase show increased sensitivity to MPTP toxicity, which is accompanied by increases in free radical production. We also found that transgenic mice with a mutation associated with familial ALS showed increased sensitivity to MPTP toxicity. These mice are known to have increases oxidative damage. These studies have, therefore, made significant progress on the original aims of the proposal.</p>				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	16
Reportable Outcomes.....	17
Conclusions.....	18
References.....	19
Appendices.....	20

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4. INTRODUCTION

Oxidative stress from endogenous and exogenous oxidants has been implicated as a major cause of Parkinson's Disease. The subject of the present research is to:

1. Investigate whether there is coherent evidence of increased oxidative damage to proteins, lipids and DNA in postmortem tissue of patients with Parkinson's Disease.
2. To develop novel HPLC based assays for quantitation of products of oxidative damage in human CSF, plasma and urine samples and to apply these biomarkers to study whether they are altered in patients with Parkinson's Disease.
3. To determine whether oxidative stress plays a key role in neuronal death, which occurs in the MPTP model of Parkinson's Disease and whether the same stress may be operating in vulnerable human nigral neurons.

In particular, we were going to examine whether transgenic mice with alterations in free radical scavenging enzymes or which overexpress Bcl2 are resistant to MPTP neurotoxicity. We were also going to examine whether free radical spintraps, neuronal nitric oxide synthase inhibitors and creatine can block MPTP neurotoxicity. Finally, we were going to examining the expression of genes encoding proteins which may determine vulnerability to oxidative stress in human dopamine neurons.

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5. BODY

The program has now continued in two phases centered at the Weill Medical College of Cornell University and at the Massachusetts General Hospital. Dr. Anne B. Young, Chief of Neurology at Massachusetts General Hospital is now supervising the studies, which are ongoing there. Work at the Massachusetts General Hospital over the past year has focused on two of the Technical Objectives of the study:

Objective #1 – to determine whether there is coherent evidence of increased oxidative damage to the protein, lipid and/or DNA fractions in postmortem human brain tissue of patients with PD as compared to age-matched controls and whether this damage specifically occurs in the vulnerable substantia nigra dopamine neurons.

As described in previous progress reports, we have adopted a new technology, Laser Capture Microdissection, which allows us to address these questions more directly than the methods described in the original proposal. Two specific approaches have been used: 1) direct analysis of oxidative damage to DNA in the mitochondrial of dopaminergic neurons, and 2) analysis of gene expression in dopamine neurons.

1) Direct analysis of oxidative damage to the mitochondrial DNA of dopamine neurons.

Progress in this area has been greatly aided by a recently formed collaboration with Dr. David Simon, MD, PhD of the Beth Israel –Deaconess Medical Center in Boston.

It has been previously demonstrated that brain levels of 8-hydroxy-2'deoxyguanosine (OH⁸dG), a marker of oxidative DNA damage associated with point mutations, are 16-fold higher in mitochondrial DNA (mtDNA) than in nuclear DNA, increase with aging, and increase further in PD. The locus of damaged DNA in the brain is uncertain. Dopaminergic neurons are the principal target of neurodegeneration in PD. Oxidative stress due to dopamine catabolism may contribute to susceptibility of dopaminergic neurons to the accumulation of acquired mtDNA mutations. However, oxidative damage and acquired mtDNA mutations may not be limited to neuronal cells, but instead could also accumulate in glia.

The oxidation of guanidine bases to form OH⁸dG can in turn can induce mutations during DNA replication, as OH⁸dG can pair with adenine as well as cytosine with almost

equal efficiencies. This results in G:C to T:A or T:A to G:C mutations. However, direct detection of these mutations in mtDNA has not been accomplished and may not be possible by standard techniques, particularly if each specific mutation is present at a very low frequency. This situation of multiple different individually rare mtDNA mutations induced by oxidative damage would be undetectable by conventional sequencing methods, which detect alterations only when a specific mutation (at a particular base pair) is present in a large percentage of the mtDNA molecules. More sensitive techniques, such as two-dimensional denaturing gradient gel electrophoresis, still may lack the sensitivity required to detect this situation. However, these individually rare mutations can be detected by isolating and clonally expanding individual mtDNA species. Sequencing of many clones from the same source allows an estimation of the frequency of acquired mtDNA mutations in the original mtDNA source. The combination of this cloning strategy with Laser Capture Microdissection (LCM) allows these questions to be addressed for specific neuronal and glial populations in PD

Laser Capture Microdissection

Using the Arcturus PicCell II instrument, we have developed protocols for the identification and isolation of neurons and glia from human brain. These methods employ 8 μ m thick section prepared from frozen tissue blocks. Dopamine neurons in the substantia nigra can be readily identified in unstained tissue by the presence of neuromelanin, and reliably captured, as illustrated below. Glia can be identified by the presence of glial fibrillary astrocytic protein (GFAP) or other markers through immunohistochemical labeling (see Methods below).

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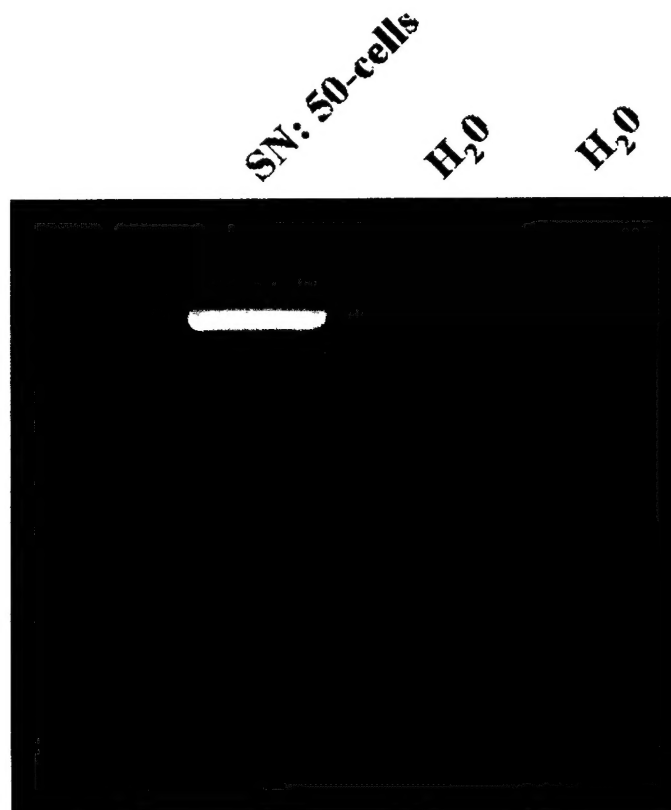
Isolation of dopamine neurons by LCM. A single melanized neuron (a) is visualized and then removed by LCM (b) and can be seen isolated on the EVA film (c) after dissection.



PCR amplification of LCM-collected neurons

Using a nested PCR protocol (see methods), we are able to amplify a mtDNA fragment from DNA isolated from low numbers of LCM-collected neurons. Shown below are the results of PCR amplification of the ND4 gene from DNA isolated from 50 LCM-collected neurons (second lane) demonstrating a PCR band of the predicted approximately 1 kilobase size with no secondary bands. The left lane is a 100 base-pair ladder. We have obtained similar results with samples containing as few as 10 LCM-collected neurons. Absence of any band in the right lanes provides evidence against contamination. However, because contamination might occur sporadically, we do not consider this alone to be sufficient control for this possibility.

PCR amplification of LCM-collected neurons



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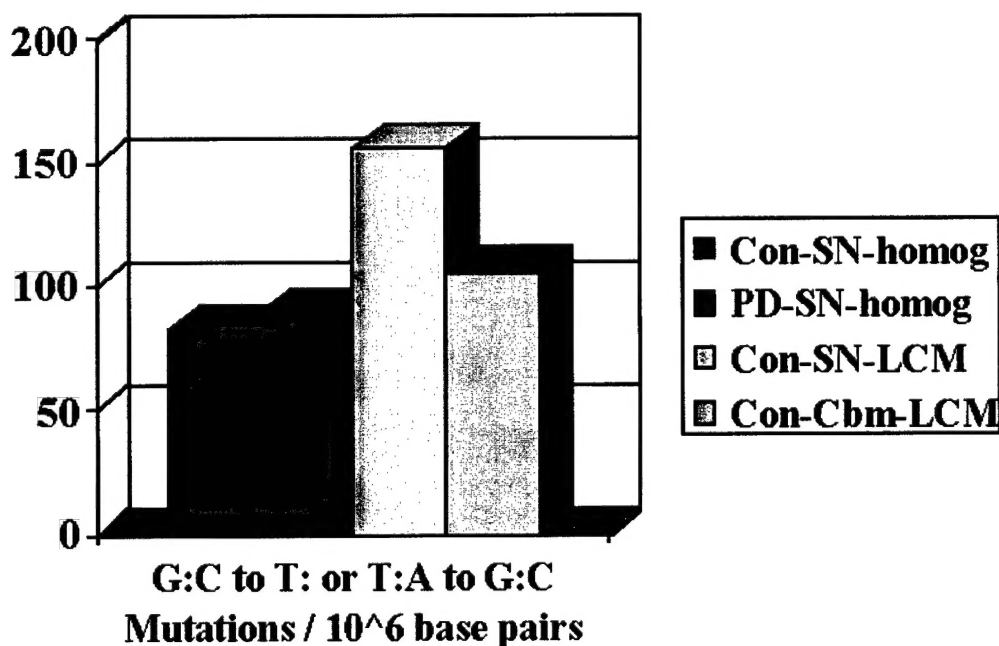
Polymorphisms present in the region of mtDNA under study provide an important additional strategy to rule out contamination. When a homoplasmic polymorphism is present in the D-loop or the ND4 gene in a subject, then the presence of this polymorphism in each of the sequenced D-loop or ND4 gene clones from this subject is used to verify that the origin of the cloned DNA is that subject. For example this individual had the following homoplasmic ND4 gene polymorphisms: T11299C, A11467G, and T11485C, based on direct sequencing (without cloning) of DNA isolated from brain dissected by standard methods. The first two polymorphisms are relatively common, but the third, at position 11485, is a homoplasmic synonymous polymorphism (no alteration of the encoded amino acid) that has not previously been reported. DNA was isolated from 100 cerebellar granule neurons collected by LCM, PCR amplified, and cloned. DNA isolated from 38 of these clones was sequenced. Each of these three polymorphisms, including T11485C, was present in each of the 38 clones, providing further verification that the amplified DNA originated from this subject rather than from contaminant DNA. Analysis of the D-loop will allow this strategy to be used in a many subjects, as the D-loop is contains highly polymorphic sites. Allele specific PCR provides another mechanism for avoiding contaminating DNA (see methods: PCR amplifications).

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Mutational Analysis of mtDNA from Dopamine neurons

The results of mutational analysis of mtDNA from laser-capture isolated neurons

Oxidative Stress-induced MtDNA Mutations



is illustrated below. Also shown is data from analysis of samples prepared by conventional homogenization of the tissues, for comparison. Data are expressed as mutations $\times 10^{-6}$ per base pair. In homogenized substantia nigra from control or PD cases, the observed rate of oxidative mutation is similar. However, analysis of the LCM captured neurons from control substantia nigra reveals that the mtDNA of these cells has a much higher mutation rate than is observed in tissue homogenates or in neurons from the cerebellum. These observations are based on 54 clones from the control SN neurons, with 31,944 base pairs sequenced. The cerebellar granule neuron data is based on 62 clones, with 28,140 base pairs sequenced.

These data, although preliminary in nature, suggest several important points:

- 1) measurement of oxidative damage to mtDNA by this technique is very sensitive; indeed, we think it will be possible to study single dopamine neurons.
- 2) The mutation rate in isolated dopamine neurons is much higher than that measured in tissue extracts. This implies selective accumulation of mutations in neurons, in contrast to glia and other tissue components. We will be able to test this directly by laser capture of glial cells for analysis.

At present we have captured neurons from human Parkinson's disease neurons. We were also able to isolate mtDNA from these, and sequence analysis is currently in progress.

Specific Methods for Mutational Analysis

Immunohistochemistry: To identify dopaminergic neurons, brain sections are fixed with 70% ethanol then stained rapidly with methylene blue (0.1% for 20 seconds) and dehydrated through increasing concentrations of ethanol and xylene.

Immunohistochemistry for the different glial markers will be performed on the frozen slices for identification of astrocytes. 8 μ m frozen sections will be fixed in acetone; rehydrated in RNase-free water (30 sec), incubated with the primary antibody for 10min at room temperature and developed with either the Vectastain universal quick kit (universal biotinylated secondary antibody for 10 min, followed by a streptavidin/peroxidase complex for 5 min, and DAB for 5 min), or Cy3-conjugated secondary antibody for 10 min. Slides stained with either methods will then be dehydrated to xylene and dried.

Laser Capture Microdissection: Laser Capture Microdissection is performed with an Arcturus PixCell II instrument located in Dr. Standaert's laboratory at the MGH. Briefly, the stained section is placed on an inverted microscope and overlaid with a thermoplastic polymer (EVA) transfer cap. The cells are selected for laser-dissection with an adjustable laser beam and a laser pulse melts the EVA film onto the targeted selected cell where it solidifies. The cells captured are then harvested by removing the cap from the tissue section. The cap is then fitted onto a sterile 0.5 ml microcentrifuge tube containing DNA extraction buffer, inverted and placed on ice for 20 min. Finally, the microcentrifuge tube and EVA cap are centrifuged and the cap is removed. The cellular content of the selected cells is now in solution. A standard phenol/chloroform extraction is used to purify the DNA, which is subsequently precipitated with ethanol in the presence of glycogen as a carrier.

PCR amplification: PCR amplification is performed with the Advantage HF-2 PCR Kit (Clontech). This kit includes a proofreading polymerase and results in 25-fold increased fidelity compared to standard Taq. The HF-2 Kit leaves "A overhangs" (as required for TA cloning) in about 25% of amplified PCR molecules. This provides sufficient efficiency for TA cloning for the proposed studies. Two pre-cloning PCR reactions are performed, with .01 μ l of the product of the first PCR reaction being used as the template for the second PCR reaction. Each of these PCR reactions follows the same PCR protocol used for our prior control experiments that ruled out PCR errors and nuclear pseudogenes as complications for this study. Primers used in the second PCR reaction are internal to those of the first PCR reaction to optimize PCR efficiency. M13 (forward and reverse) plasmid primers are used in the post-cloning PCR reactions. In some cases, *allele-specific PCR* will be used as a mechanism for avoiding contaminating DNA. An oligonucleotide is designed that is mispaired with 1-3 base pairs at its 3' end, but the terminal base pair (at the 3' end is designed to pair appropriately with a homoplasmic polymorphism present in the DNA of interest. PCR parameters (annealing temperature; number of cycles, etc...) can be optimized to allow specific amplification of DNA bearing this polymorphism without amplification of any DNA that lacks the polymorphisms. This method can be used for any subjects harboring at least two relatively uncommon polymorphisms: one for use in designing the allele-specific PCR, and one for verification of the origin of the cloned DNA. Samples with the required polymorphisms have been identified and allele specific primers are now being optimized for this purpose. Optimization involves systematic variation of annealing temperature

and cycle numbers to achieve optimum specificity. This method provides a further mechanism to ensure that only the DNA of interest will be amplified.

Cloning: Cloning of individual mtDNA molecules is performed using the TOPO TA Cloning Kit (Invitrogen) according to the manufacturers directions. Mixing of mtDNA fragments from two sources known to differ at a polymorphic site verifies that each clone originates from a single mtDNA molecule, as none of the resulting clones show heteroplasmy for the polymorphic site (data not shown).

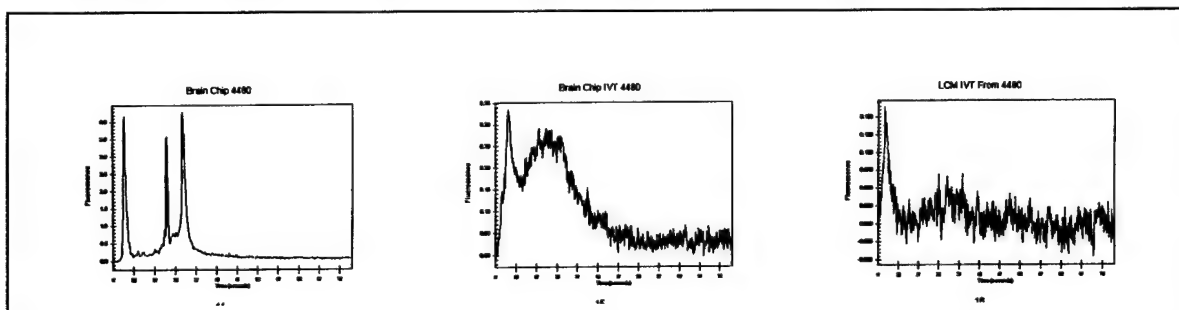
Mutation identification: Sequence Navigator software (Perkin Elmer) is utilized for computerized multiple sequence alignment with automatic highlighting of discrepancies compared to a reference sequence. This avoids the potential for errors that can result from "hand"-analysis of sequence data, and allows rapid analysis of large volumes of data. Each mutation highlighted is compared with the corresponding region of the original electropherogram to ensure that it is not an artifact related to "noise". The sequencing methods used here consistently give extremely low-noise results that allow highly reliable mutation identification of 400 to 600 base pairs or more for each sequencing reaction.

Analysis of gene expression in dopamine neurons.

In addition to the direct detection of mtDNA mutations in human dopamine neurons, we are also using the laser capture technique to isolate mRNA for analysis of gene expression for dopamine neurons. The rationale for this approach is described in detail in previous progress reports.

The principal technical barrier to these studies has been obtaining sufficient RNA for array studies. We have worked extensively on methods of RNA amplification in the last year, along with others in the field. Within recent months we have begun to obtain reliable amplification of the mRNA isolated from laser captured neurons.

An important aspect of the procedure is verification of the success of amplification by direct analysis of the RNA. For this we have begun to use the Agilent 2100 Bioanalyzer. This instrument provides direct RNA analysis of samples as small as 5 ng. Illustrated below is analysis of three such RNA samples. The first is RNA isolated from a postmortem human brain. Two peak corresponding to ribosomal subunits are the dominant features. The second is the result of T7-based amplification of this RNA, showing a broad peak of species. Since only mRNA and not rRNA are amplified, this is



the expected result. The third trace illustrates amplification of LCM captured RNA. The yield is lower than with RNA isolated by conventional techniques, but still is adequate for array studies.

The development of suitable protocols for array analysis of human dopamine neurons has proceeded more slowly than planned, and we do not yet have sufficient array data to report a result. However, many of the technical barriers have been surmounted in recent months, and we expect to move forward rapidly at this point.

We are continuing studies using immunocytochemical assays for oxidative damage from Parkinson's Disease postmortem tissue. This is being done on a panel of human brain tissue from normal midbrain as well as a smaller number of samples from patients with Parkinson's Disease.

Objective #2: To develop novel HPLC based assays for quantitation of products of oxidative damage in human CSF, plasma and urine samples and to apply these biomarkers to study whether they are altered in patients with Parkinson's Disease.

We have developed a novel column switching assay to measure 8-hydroxy-2-deoxyguanosine concentrations in urine, plasma and CSF as well as other biological

matrices. This is an unique methodology, which was recently published in detail. We have recently utilized this technology to assess OH⁸dG levels in samples from patients with ALS. We have been successful in demonstrating for the first time that OH⁸dG levels are significantly elevated in the CSF, plasma and urine of ALS patients. We are continuing to collect samples from Parkinson's Disease patients to carry out similar measurements. We have carried out initial measurements in plasma samples of 12 Parkinson's Disease patients and 7 controls. These studies showed no significant differences. The mean values in controls were 26.1 ± 3.2 and in Parkinson's Disease 25.5 ± 4.1 ng/ml. We are continuing these studies but they may indicate that increased oxidative damage in the central nervous system may not be reflected in the plasma.

We have now established an assay for nitro-gammatocopherol for use in human body fluids. We have utilized this in 6 normal samples and have demonstrated a consistent ability to detect this compound. It requires a hexane extraction of plasma in which an internal standard for retinol acetate and COQ9 are added. The hexane extracted sample is then dried down and reconstituted in a mobile phase consisting of 50% methanol and 50% ethanol. Samples are then run by HPLC using a 12 electrode coularray system. Utilizing this methodology we can then measure α -tocopherol, gamma-tocopherol, nitro-gammatocopherol and CoQ10 levels. We will utilize this methodology for examining whether there is evidence of increased nitration mediated by peroxynitrite in plasma samples of Parkinson's Disease patients as compared to normal controls.

During the past year, we have continued to study the parkinsonian syndrome Progressive Supranuclear Palsy. We have carried out further studies of cybrids obtained from platelets of Progressive Supranuclear Palsy patients. We had previously demonstrated that the cybrids show a significant reduction in α -ketoglutarate dehydrogenase activity in post mortem brain tissue. In the studies of the cybrids we further examined whether there is evidence of mitochondrial dysfunction. Cybrids are made from taking cells which have no mitochondria and then fusing the patient's platelet mitochondria to place them in a different nuclear background. If one then observes a defect in mitochondrial function, it implies that it was encoded on the mitochondrial genome. We observed significant decreases in aconitase activity, cellular ATP levels and oxygen consumption in Progressive Supranuclear Palsy cybrids, as compared to control cybrids. These findings provide further evidence that increase free radical production of mitochondrial dysfunction may play a critical role in the pathogenesis of

Progressive Supranuclear Palsy. This again provides further evidence that a parkinsonian syndrome has a prominent role of oxidative damage in its pathogenesis.

Objective #3: to determine whether oxidative stress plays a role in the neuronal death in the MPTP model of Parkinson's disease, and whether the same stress may be operating in human nigral neurons.

We have carried out further studies examining the role of oxidative damage in MPTP toxicity. In particular, we have examined MPTP toxicity in mice, which have a deficiency in the free radical scavenging enzyme manganese superoxide dismutase. This enzyme is of critical importance in modulating oxidative damage in the central nervous system. Mice, which have a total deficiency of the enzyme, die prematurely with vacuolar neuronal degeneration. We examined the effects of 50% deficiency of this enzyme. We have determined that the heterozygous manganese superoxide dismutase knockout mice show no evidence of neuropathological or behavioral abnormalities at 2 – 4 months of age. These mice show significantly increased vulnerability to dopamine depletion, following the administration of MPTP. There were also significantly greater reductions in the dopamine metabolites DOPAC and HVA as compared with wild type mice. We also examined the effects on neurons in the substantia nigra compacta. There was a significantly greater reduction in both Nissl and dopamine transporter protein neurons in the MPTP treated SOD2 deficient mice. We also examined the effects of two other mitochondrial toxins in these mice. We examined both 3-nitropropionic acid and malonate. Both of these toxins produced significantly larger lesions in the mice with a partial deficiency of manganese superoxide dismutase.

Lastly, we determined whether these mice showed increased production of hydroxyl radicals after malonate injections as measured with the salicylate hydroxyl radical trapping method. We found that there was a significant increase in the conversion of salicylate to 2,5 dihydroxybenzoic acid from salicylate in these mice. We have also carried out further studies of MPTP toxicity in transgenic mice, which carry the G93A mutation in copper zinc superoxide dismutase which is associated with familial ALS. These mice develop progressive weakness and degeneration of the motor neurons in the spinal cord. They also interestingly have a delayed onset degeneration of substantia nigra neurons. We previously demonstrated that there is increased production of oxidative damage in these mice. We found that administration of MPTP to

these mice resulted in a significantly greater depletion of dopamine at 50 and 70 days of age, which is prior to the onset of loss of dopaminergic neurons. There was also a significant increase in the toxicity of the mitochondrial toxins 3-nitropropionic acid. There was a slightly greater loss of tyrosine hydroxylase neurons in these mice in response to MPTP, however, the result did not quite reach significance as compared to littermate controls.

6. KEY RESEARCH ACCOMPLISHMENTS

- A. The demonstration that one can directly measure mitochondrial DNA mutations in isolated human substantia nigra neurons.
- B. The application of measurements of 8-hydroxy-2-deoxyguanosine in human plasma to patients with Parkinson's Disease.
- C. The finding of impaired mitochondrial function in cybrids from the Parkinsonian syndrome Progressive Supranuclear Palsy.
- D. The finding that mice which are deficient and the antioxidant enzyme manganese superoxide dismutase show increased vulnerability and increased evidence of oxidative damage produced by MPTP.
- E. The finding that mice with a mutation in copper/zinc superoxide dismutase which is associated with familial ALS show increased vulnerability to MPTP.

7. REPORTABLE OUTCOMES

Andreassen OA, Ferrante RJ, Dedeoglu A, Albers DW, Klivenyi P, Carlson E J, Epstein CJ, Beal MF. Mice with a partial deficiency of manganese superoxide dismutase show increased vulnerability to the mitochondrial toxins malonate, 3-nitropropionic acid, and MPTP. *Exp. Neurol* 2001; 167: 189-195.

Albers DS, Swerdlow RH, Manfredi G, Gajewski C, Yang L, Parker Jr WD, Beal MF. Further evidence for mitochondrial dysfunction in progressive supranuclear palsy. *Exp. Neurol.* 2001;168:196 - 198.

Andreassen OA, Ferrante RJ, Klivenyi P, Klein AM, Dedeoglu A, Albers DS, Kowall NW, Beal MF. Transgenic ALS mice show increased vulnerability to the mitochondrial toxins MPTP and 3-nitropropionic acid. *Exp. Neurol* 2001; 168:356-363

Tarnopolsky MA, Beal MF. Potential for creatine and other therapies targeting cellular energy dysfunction in neurological disorders. *Annals of Neurol* 2001;49: 561-574.

Beal MF. Experimental models of Parkinson's Disease. *Nature Reviews/Neuroscience* 2001;2:326-332

8. CONCLUSIONS

We have made substantial progress in demonstrating that there are increased markers of oxidative damage in post mortem brain tissue. We have now demonstrated the feasibility of directly measuring mitochondrial DNA mutations, which are a consequence of oxidative damage and isolated substantia nigra neurons. We have developed a highly sensitive assay for measurement of oxidative damage to DNA and have applied this to Parkinsonian patients.

We have found that a number of novel genetic manipulations can markedly increase both oxidative damage and dopaminergic neurotoxicity in the MPTP model of Parkinsonism. In particular, we have demonstrated that mice with the human copper/zinc SOD1 mutation associated with familial ALS show increased sensitivity to MPTP. This is of interest since it shows that a combination of genetic defect with an environmental toxin can produce selective substantia nigra damage.

These studies greatly strengthen the implication of oxidative damage in Parkinson's Disease pathogenesis.

9. REFERENCES

None.

10. APPENDICES

One copy of each of the cited papers in reportable outcomes.

Mice with a Partial Deficiency of Manganese Superoxide Dismutase Show Increased Vulnerability to the Mitochondrial Toxins Malonate, 3-Nitropropionic Acid, and MPTP

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There is substantial evidence implicating mitochondrial dysfunction and free radical generation as major mechanisms of neuronal death in neurodegenerative diseases. The major free radical scavenging enzyme in mitochondria is manganese superoxide dismutase (SOD2). In the present study we investigated the susceptibility of mice with a partial deficiency of SOD2 to the neurotoxins 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), 3-nitropropionic acid (3-NP), and malonate, which are commonly used animal models of Parkinson's and Huntington's disease. Heterozygous SOD2 knockout (SOD2^{+/-}) mice showed no evidence of neuropathological or behavioral abnormalities at 2–4 months of age. Compared to littermate wild-type mice, mice with partial SOD2 deficiency showed increased vulnerability to dopamine depletion after systemic MPTP treatment and significantly larger striatal lesions produced by both 3-NP and malonate. SOD2^{+/-} mice also showed an increased production of "hydroxyl" radicals after malonate injection measured with the salicylate hydroxyl radical trapping method. These results provide further evidence that reactive oxygen species play an important role in the neurotoxicity of MPTP, malonate, and 3-NP. These findings show that a subclinical deficiency in a free radical scavenging enzyme may act in concert with environmental toxins to produce selective neurodegeneration. © 2001 Academic Press

Key Words: MPTP; malonate; free radicals; 3-nitropropionic acid; Parkinson's disease; Huntington's disease.

INTRODUCTION

The mechanism of nerve cell death in neurodegenerative diseases is being extensively studied. A central

role for oxidative damage in the pathogenesis of Parkinson's disease (PD) and Huntington's disease (HD) is gaining increasing acceptance (3, 4, 16). There is also substantial evidence linking mitochondrial dysfunction and free radical formation (4). Mitochondria consume more than 90% of the cell's oxygen, and the respiratory chain in the mitochondria is a major source of superoxide radicals (14).

There are two intracellular superoxide dismutase enzymes. The copper- and zinc-containing superoxide dismutase (SOD1) is mainly located in cytoplasmic and nuclear compartments. The manganese-containing superoxide dismutase (SOD2) is localized predominantly in the mitochondria (10, 34). A third superoxide dismutase, also containing copper and zinc, is found mainly extracellularly (28). The SOD2 enzyme plays a critical role in normal antioxidant defense. Homozygous knockout mice which are deficient in SOD2 develop myocardial injury and neurodegeneration and die perinatally (24, 26). Hemizygous knockout mice with a partial deficiency show increased superoxide radical levels and larger brain lesions after focal ischemia (30).

A commonly used neurotoxin model for PD is 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP). There is substantial evidence that MPTP neurotoxicity involves inhibition of mitochondrial function in which the formation of the toxic metabolite 1-methyl-4-phenylpyridinium (MPP⁺) and its accumulation in mitochondria plays a major role (33). This may result in a pathologic cascade, leading to free radical generation and excitotoxicity (22, 32). The neurotoxins malonate and 3-nitropropionic acid (3-NP) have been well characterized as experimental models of HD (6, 21). These toxins inhibit succinate dehydrogenase, inducing mito-



chondrial dysfunction which triggers the generation of superoxide radicals and secondary excitotoxicity (5).

Hemizygous mice deficient in SOD2 (SOD2^{+/-}) have an approximate 50% decrease in SOD2 activity (25, 36). These mice show no evidence of neuropathological or behavioral abnormalities at 2–4 months of age. Crossing these mice into transgenic amyotrophic lateral sclerosis (ALS) mice with SOD1 mutations (G93A) significantly exacerbates the onset of symptoms and decreases survival (1). In the present study, we examined the susceptibility of SOD2^{+/-} mice to MPTP, malonate, and 3-NP neurotoxicity.

MATERIALS AND METHODS

Male hemizygous SOD2 knockout mice (SOD2^{+/-}) (25) were bred with females with the CD-1 background strain (Charles River, Wilmington, MA). The F1 generation of SOD2^{+/-} mice used in the experiments was genotyped using PCR analysis of tail DNA. All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee.

MPTP (Research Biochemicals, Wayland, MA) was dissolved in phosphate-buffered saline (PBS) and 15 mg/kg was injected ip in a volume of 0.15 ml every 2 h for five doses in wild-type littermate control and SOD2^{+/-} mice. Control mice received PBS vehicle. Eight to ten animals were examined in each group. Mice were sacrificed 7 days after MPTP injections and the striata were dissected and placed in chilled 0.1 M perchloric acid. Samples were sonicated and centrifuged, and an aliquot was taken for measurements of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) by HPLC, using electrochemical detection as previously described (9).

In a similar experimental paradigm, histopathological evaluation of the effects of MPTP ip injections was performed in 10 wild-type littermate control and 10 SOD2^{+/-} mice. Both PBS-treated wild-type and SOD2^{+/-} mice were also examined. The mice received the same treatment regimen as above. Seven days after the last MPTP injection the mice were deeply anesthetized and transcardially perfused with phosphate-buffered 4% paraformaldehyde. The brains were postfixed for 24 h and cryoprotected in a graded series of 10 and 20% glycerol/2% DMSO solution. Tissue specimens were subsequently serially cut on a cryostat at 50 μ m, stored in six separate collection wells, and stained for Nissl using cresyl violet as previously described (17). Cut tissue sections of the midbrain were immunostained for dopamine transporter (DAT) (DAT antisera, 1:500 dilution; Chemicon International, Inc). DAT labels neurons of the substantia nigra pars compacta (11). Midbrain sections through both the left and the right substantia nigra from the bregma levels \bar{n} 3.08 to \bar{n} 3.16 mm and intraaural levels 0.72 to 0.64

mm (19) were analyzed by microscopic videocapture. Stereologic counts of Nissl- and DAT-positive neurons within the substantia nigra pars compacta were computed using Neurolucida (Microbrightfield, Colchester, VT) image analysis software. The dissector counting technique was employed, in which all neurons were counted in an unbiased selection of serial sections in a defined volume of the spinal cord and substantia nigra (35).

To determine whether MPTP uptake or metabolism was altered, MPTP 15 mg/kg was administered ip five times at 2-h intervals and mice were sacrificed at 1 h after the last dose ($n = 6$ /group). Striatal tissue was dissected and samples were sonicated in 0.1 M perchloric acid. MPP⁺ levels were quantified by HPLC with UV detection at 295 nm. An aliquot of supernatant was injected onto a Brownlee Aquapore X03-224 cation exchange column (Rainin, Woburn, MA). Samples were eluted isocratically with 90% 0.1 M acetic acid and 75 mM triethylamine HCl, pH 2.3, adjusted with formic acid and 10% acetonitrile.

Malonate (Sigma, St. Louis, MO) was dissolved in PBS and the pH adjusted to 7.4 with HCl. Intrastriatal injections of 0.6 μ l containing 1.5 μ mol of malonate were made within the left striatum 0.5 mm anterior to bregma, 2.1 mm lateral to the midline, 3.8 mm ventral to the skull surface of both wild-type littermate control and SOD2^{+/-} mice, using a 10- μ l Hamilton syringe fitted with a 26-gauge needle. Animals were euthanized 7 days after the injection. The brains were quickly removed and placed in an ice-cold 0.9% saline solution. Brains were then sectioned at 1-mm inter-

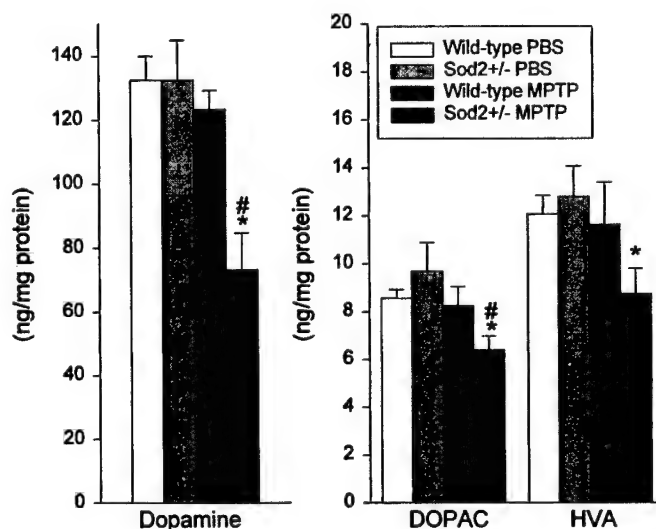


FIG. 1. The effects of MPTP (15 mg/kg, ip $\times 5$) on striatal dopamine, DOPAC, and HVA in wild-type littermate controls and SOD2^{+/-} mice. Controls received PBS. Means \pm SEM are presented ($n = 8$ –10 per group). * $P < 0.01$ compared with SOD2^{+/-} treated with PBS, # $P < 0.01$ compared to wild-type treated with MPTP (ANOVA with Fisher PLSD post hoc test).

TABLE 1

Nissl and Dopamine Transporter Protein (DAT) Neuronal Counts in the Substantia Nigra Compacta of MPTP-Treated Partially Deficient Transgenic SOD and Littermate Control Mice

	Nissl	DAT
SOD2-deficient MPTP-treated	111 \pm 10.2	78 \pm 7.2
Littermate control mice MPTP-treated	119 \pm 11.4	85 \pm 6.6
Littermate control mice	157 \pm 5.8	115 \pm 4.7

Note. Nissl- and dopamine transporter-positive neurons within the substantia nigra compacta were analyzed by videomicroscopic capture of tissue sections and image analysis software (Microbrightfield, Colchester, VT). While there was a trend toward fewer neurons in the SOD2 treated mice, there was no significant difference between them and the control MPTP-treated mice. Data are means \pm SEM.

vals. Slices were placed posterior side down in 2% 2,3,5-triphenyltetrazolium chloride. Slices were stained in the dark at room temperature for 30 min

and then removed and placed in 4% paraformaldehyde, pH 7.3. Lesions, noted by pale staining, were evaluated on the posterior surface of each section using an image analysis system (Version 5.0, Rev. 2.0, Image Research, Inc., Canada) by two independent observers blinded to experimental conditions (O.A.A. and A.D.). Eight mice were examined in each group after two brains were excluded due to misplaced lesions.

The salicylate hydroxyl radical trapping method (18) was used for measuring levels of hydroxyl radicals in striatal tissue after injection of malonate as described above. Wild-type controls and SOD^{+/−} mice ($n = 8$ per group) were administered salicylate (200 mg/kg, 5 ml/kg, ip) 30 min before striatal malonate injections. Sixty minutes after malonate injection the animals were euthanized and the left and right striata were rapidly dissected from a 2-mm-thick slice on a chilled glass plate and immediately frozen at -70°C . The samples were thawed in 0.25 ml of chilled 0.1 M perchloric acid, sonicated, and centrifuged twice. Salicylate and its me-

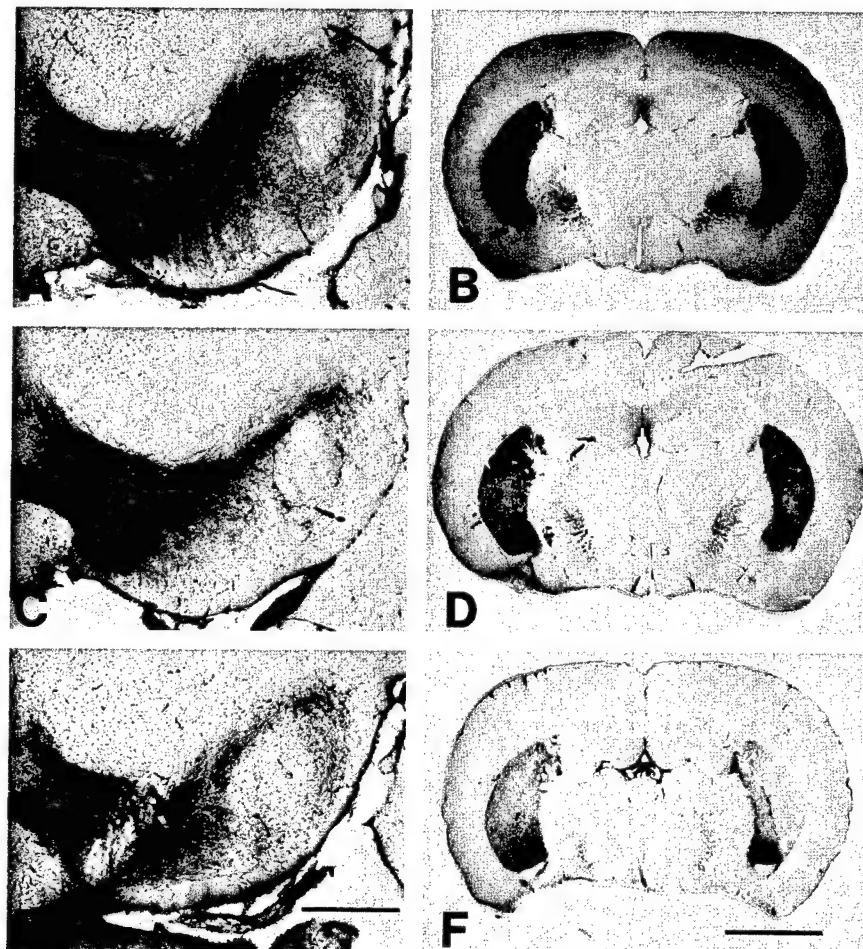


FIG. 2. Photomicrographs of dopamine transporter (DAT) immunoreactivity within the substantia nigra and neostriatum, respectively, from a littermate wild-type control mouse (A and B) and littermate wild-type control (C and D) and SOD^{+/−} (E and F) mice treated with MPTP. DAT immunoreactivity within the substantia nigra and neostriatum of MPTP-treated SOD^{+/−} mice is markedly less intense than that observed in the MPTP-treated littermate wild-type control mice. Bar in E, 500 μm and the same in A and C. Bar in F, 2 mm and the same in D and B.

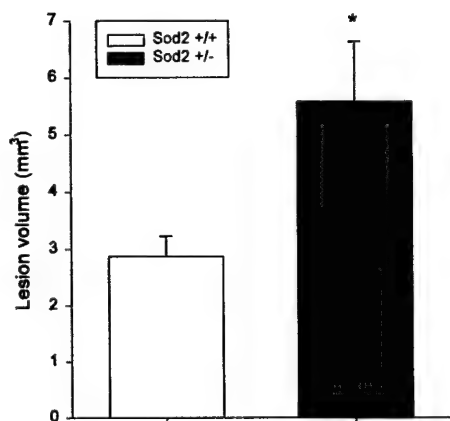


FIG. 3. Malonate lesion size after intrastriatal injection of malonate (1.5 μ mol) in SOD^{+/-} mice versus wild-type littermate controls. The means \pm SEM are presented ($n = 8$ per group). * $P < 0.05$.

tabolites 2,3- and 2,5-dihydroxybenzoic acid (DHBA) were quantified in the supernatant by HPLC with electrochemical detection (13). Data (mean \pm SEM) were expressed as the ratio of 2,3- and 2,5-DHBA to salicylate to normalize for varying brain concentrations of salicylates.

3-Nitropropionic acid (Sigma) was dissolved in PBS (pH adjusted to 7.4) and injected intraperitoneally nine times at 12-h intervals, using a dose of 50 mg/kg per injection in both SOD2^{+/-} and littermate wild-type mice ($n = 10$ for each group). The animals were deeply anesthetized and transcardially perfused with buffered 4% paraformaldehyde 3–5 h after the last 3-NP injection and processed for histopathology as above. Striatal lesion volumes were determined on Nissl-stained sections from areas of pallor in which there was increased gliosis and neuronal loss (7). Stereological analysis of lesion volumes was computed from serial sections through the striata using Neurolucida software (Micro-brightfield). The Mann–Whitney U test was used to analyze differences between groups. One-way analysis of variance followed by the Fisher protected least significance difference test was used to analyze neurochemical data.

RESULTS

As shown in Fig. 1, the concentrations of striatal dopamine, DOPAC, and HVA in SOD2^{+/-} MPTP-treated mice were significantly reduced compared to MPTP-treated littermate controls. There were no significant differences in striatal dopamine, DOPAC, or HVA concentrations following administration of PBS in wild-type littermate control and SOD2^{+/-} mice. Histopathologic evaluation of MPTP-treated SOD2^{+/-} and wild-type littermate control mice showed a greater loss of Nissl-stained and DAT-immunostained neurons within the substantia nigra compacta of SOD2^{+/-} mice;

however, statistical significance was not obtained (Table 1). In addition to the trend of fewer DAT-immunopositive neurons, DAT immunoreactivity in substantia nigra compacta neurons in SOD2^{+/-} MPTP-treated mice was less intense than that observed in MPTP-treated littermate wild-type controls, consistent with the reductions in dopamine found in the neostriatum (Fig. 2). The increased sensitivity to MPTP was not caused by an alteration in uptake or metabolism of MPTP to MPP⁺ because striatal MPP⁺ levels did not significantly differ at 1 h after MPTP administration (MPP⁺ 123.7 \pm 13.5 ng/mg in controls and 133.6 \pm 9.7 ng/mg in SOD2^{+/-} mice).

As shown in Fig. 3, the size of the malonate-induced striatal lesions in SOD2^{+/-} mice was 48.6% larger than the lesion size of littermate wild-type controls ($P < 0.04$). No pathologic alterations were observed in the PBS-injected striata other than those associated with the needle track. Following the administration of salicylate, intrastriatal injection of malonate resulted in a significant increase in the levels of 2,5-DHBA in both wild-type controls and SOD2^{+/-} mice compared to the unlesioned side ($P < 0.001$) (Fig. 4). The level of 2,5-DHBA in the lesioned side of SOD2^{+/-} mice was significantly increased compared to the lesioned side of wild-type littermate controls ($P < 0.03$). No significant differences in the levels of 2,3-DHBA were observed between the groups.

Systemic administration of 3-NP resulted in bilateral striatal lesions in the SOD2 mice, although there were no behavioral correlates (Fig. 5). The areas of neuronal loss and gliosis, however, were significantly greater in the SOD2^{+/-} mice (Fig. 5). Lesion analysis

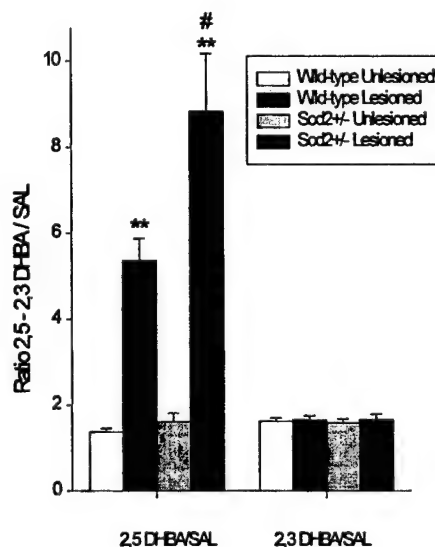


FIG. 4. The effect of intrastriatal injection of malonate (1.5 μ mol) on the conversion of salicylate to 2,3- and 2,5-DHBA in wild-type littermate controls and SOD^{+/-} mice. The ratio of 2,3- and 2,5-DHBA over salicylate is presented (means \pm SEM; $n = 8$ per group). ** $P < 0.001$ vs # $P < 0.05$ vs wild-type littermates.

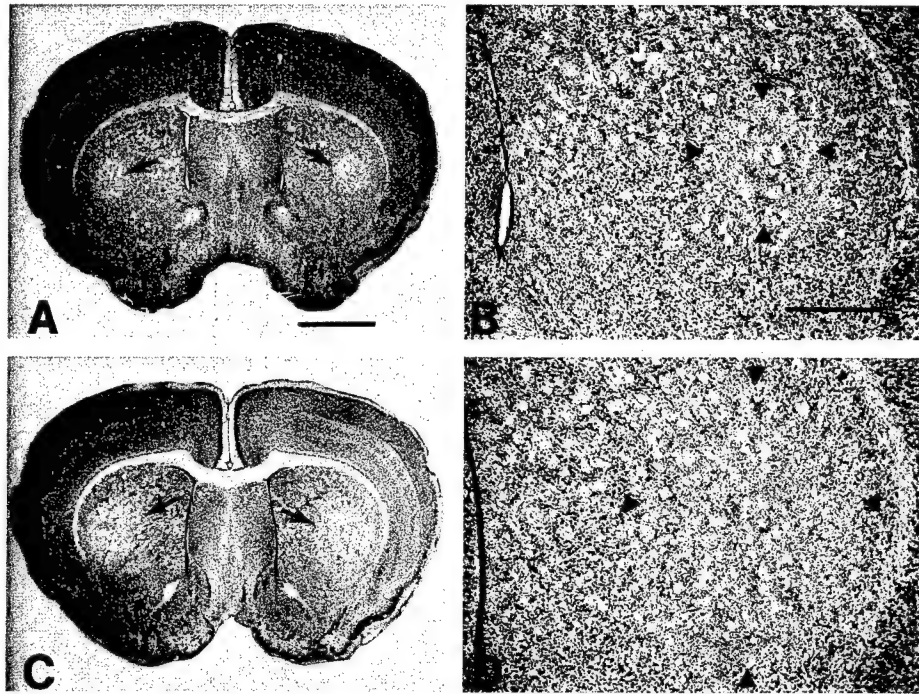


FIG. 5. Photomicrographs of 3-nitropropionic acid lesions in Nissl-stained whole brain sections through the striatum at the level of the anterior commissure of littermate wild-type (A) and SOD^{+/−} (C) mice. Bilateral striatal lesions are present in both A and B and are represented by staining pallor in the lateral aspect (arrows). The lesions are significantly larger in the SOD^{+/−} (B) than in littermate wild-type (D) mice. Bar in A, 2 mm and the same for C. Bar in B, 500 μ m and the same for D.

showed that 3-NP-induced striatal lesion volumes were significantly larger in SOD2^{+/−} mice than in littermate controls (SOD2^{+/−} mice, 15.87 ± 1.02 mm³; littermate wild-type control mice, 7.12 ± 0.61 mm³; $P < 0.001$). 3-NP-induced striatal lesion volumes were over twice as large as those found in littermate wild-type mice.

DISCUSSION

The formation of reactive oxygen species is suspected to be involved in the mechanism of nerve cell death in neurodegenerative diseases such as Parkinson's disease and Huntington's disease (4). The relative roles of endogenous and exogenous antioxidants in protecting the brain against oxidative stress are still being clarified. The antioxidant enzymes in the brain include SOD1 and SOD2, which catalyze the conversion of O₂ to H₂O₂ (20). H₂O₂ is then converted to H₂O by either catalase or selenogluthathione peroxidases. SOD2 may be particularly important since mitochondria are a major endogenous source of free radicals (14). In SOD2-deficient mice, survival can be increased by administration of SOD2 mimics; however, this results in extensive neuronal damage (29). SOD2 is also susceptible to inactivation by peroxynitrite and has been linked to kidney rejection following transplantation (27). The inactivation of SOD2 by peroxynitrite could lead to a neurotoxic cascade in which further free radical generation may progressively impair free radical defenses.

Increased nitration of SOD2 is found in cerebrospinal fluid in Alzheimer's and Parkinson's disease and it is strikingly elevated in patients with ALS (2).

In the present study, we found that SOD2^{+/−} mice have an increased vulnerability to the neurotoxins MPTP, malonate, and 3-NP. SOD2 is the primary antioxidant enzyme protecting against superoxide radicals produced within the mitochondria (20). The heterozygous SOD2^{+/−} knockout mice used in this study exhibit a 50% reduction in SOD2 levels, but have normal development and behavior. They show no neuropathological features up to 6 months of age. With increasing age they have some evidence of increased oxidative damage (36).

Malonate is a reversible inhibitor and 3-NP is an irreversible inhibitor of the succinate dehydrogenase (complex II) of the electron transport chain in the mitochondria (5, 6, 21). The energy deficits produced by these toxins lead to striatal lesions that closely resemble the neuropathology of HD (8, 15, 21). The lesions are associated with increases in markers of free radical damage and they are attenuated in mice overexpressing SOD1 (7). The oxidative phosphorylation system within the mitochondria is the major endogenous source of reactive oxygen species, which are the toxic by-products of respiration. Energy depletion also triggers activation of excitatory amino acid receptors and contributes to pathologic cascades leading to cell death (31). SOD2 activity may therefore be particularly im-

portant in protecting against the neurotoxicity of mitochondrial toxins. In the present experiments we found that malonate lesions were 50% larger in the SOD2^{+/-} mice. Similarly the lesions produced by 3-NP were twofold larger in the SOD2^{+/-} mice.

In the present study, we observed that partially deficient SOD2 mice show increased levels of hydroxyl radicals in the striatum after administration of the mitochondrial toxin malonate, consistent with the reduced free radical scavenging capacity of the partially SOD2-deficient mice, making them more vulnerable to increases in reactive oxygen species produced by the neurotoxins which act at the level of mitochondria. Our results are in accordance with an earlier study in which mice with reduced SOD2 activity showed increased superoxide radicals and larger lesions after focal ischemia (30).

MPTP is a selective dopaminergic toxin which produces cell death in the substantia nigra, similar to that observed in PD patients (12). MPTP is metabolized by monoamine oxidase B to MPP⁺ which is taken up into dopaminergic neurons by the synaptic dopamine transporter. MPP⁺ accumulates in mitochondria where it inhibits complex I of the electron transport chain (33). This may subsequently lead to indirect excitotoxicity and free radical generation (22, 32). We found that MPTP produced a greater dopamine depletion in the striatum in SOD2^{+/-} mice than littermate controls, consistent with a role of free radicals produced by mitochondria. Similarly we previously found that MPTP toxicity is attenuated in mice overexpressing SOD2, consistent with the present results (23).

These findings provide further support for a central role of defective mitochondrial energy production and the resulting increase in free radical formation in the pathogenesis of neurodegenerative disorders (4). The results are consistent with the hypothesis that subclinical genetic defects in free radical scavenging enzymes in man may act together with environmental toxins in the pathogenesis of neurodegenerative diseases.

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BRIEF COMMUNICATION

Further Evidence for Mitochondrial Dysfunction in Progressive Supranuclear Palsy

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Lichuan Yang,^{*} W. Davis Parker, Jr.,[†] and M. Flint Beal^{*}

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Recent data from our laboratory have identified a role for mitochondrial dysfunction in the pathogenesis of progressive supranuclear palsy (PSP). To extend this finding, we measured key parameters of mitochondrial function in platelet-derived cytoplasmic hybrid (cybrid) cell lines expressing mitochondrial genes from patients with PSP. We observed significant decreases in aconitase activity, cellular ATP levels, and oxygen consumption in PSP cybrids as compared to control cybrids, further suggesting a contributory role of impaired mitochondrial energy metabolism in PSP, possibly due to genetic abnormalities of mitochondrial DNA. © 2001 Academic Press

Key Words: neurodegeneration; cybrids; ATP; oxygen; aconitase.

Progressive supranuclear palsy (PSP) is a rare neurological disorder with rapid progression characterized by the appearance of supranuclear gaze palsy in addition to extrapyramidal symptoms, which usually start during the sixth decade of life (17). The etiology of this disorder is unknown; however, accumulating evidence suggests a contributory role for impaired mitochondrial function (for review, see 3). Numerous PET studies have demonstrated defects in energy metabolism in PSP and recent data from this laboratory have demonstrated a significant reduction in α -ketoglutarate dehydrogenase complex (KGDHC) activity in postmortem PSP brain (2). Defects in oxidative phosphorylation have also been reported in muscle mitochondria from PSP patients (6). Together these studies provide com-

elling evidence for mitochondrial dysfunction underlying the pathogenesis of PSP.

Several neurodegenerative disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), are associated with defects in mitochondrial function (for review, see 3). More specifically, studies with mitochondrially transformed cells (cybrids) have demonstrated that the transfer of mitochondria from affected subjects into mitochondrially depleted neuroblastoma cells recapitulates the defects supporting the hypothesis of a pathophysiologic role of mitochondria in these diseases (16, 18, 19). Cybrid cell lines containing mitochondria DNA (mtDNA) from platelets of PSP patients have recently been made (20). Thus, we have used this PSP cybrid cell system to search for evidence of mitochondrial dysfunction due to mtDNA abnormalities.

Cybrids were prepared as described previously (20). Briefly, platelets from blood samples of patients meeting NINDS-SPSP criteria for probable PSP were isolated and fused with human neuroblastoma cells depleted of mitochondrial DNA using polyethylene glycol. Controls were either spouses of the patients or spouses of patients with other movement disorders attending the same referral clinic. The mean \pm SD age for the PSP patients was 72.7 ± 8.4 years, and for the controls, it was 67.9 ± 7.5 years (not statistically significant). Males and females were represented equally in each group.

The resultant individual cybrid lines were expanded as "mixed" clonal populations, since discrete, transformed cell colonies were not isolated prior to the initial passage. After at least 6 weeks of continuous culture in selective media lacking uridine, the resultant cybrid lines were harvested for biochemical assays. All assays were performed blinded.

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Samples for aconitase activity were prepared and assayed as described previously (21). All assays were performed at room temperature. Protein determinations were performed using the Bio-Rad D_c protein assay (Hercules, CA), using bovine serum albumin as standard. Data are expressed in nanomole per minute per milligram protein.

Samples for determination of ATP levels were prepared and assayed using sensitive high-performance liquid chromatography (HPLC) with UV detection as described previously (10). Briefly, cell pellets were homogenized in 0.4 M perchloric acid and the supernatants were neutralized with 4 M K₂CO₃ and recentrifuged. Supernatants from the second centrifugation were stored at -80°C until injected in parallel. Quantitation of ATP levels was based on integration of peak area and compared with ATP standards. Protein determinations were performed using the Bio-Rad D_c protein assay using bovine serum albumin as standard. Data are expressed in nanomoles of ATP per milligram protein.

Measurement of oxygen consumption by intact cells was performed as described elsewhere (8) using Oxo-1 Clark-type computerized oxygraph (Hansatech, Norfolk, UK). Cells grown in DMEM containing high glucose and pyruvate were trypsinized and resuspended at 1.5×10^6 cells/ml in DMEM containing only 110 mM pyruvate. Equal aliquots of the different cell suspensions were used to measure oxygen consumption and the reaction was arrested by the addition of 80 mM potassium cyanide. Data are expressed in nanomole O₂ per minute.

Results are presented as means \pm standard error of the mean (SEM). Statistical comparisons between PSP and control values were made using the Mann-Whitney *U* test (InStat, GraphPad, San Diego, CA).

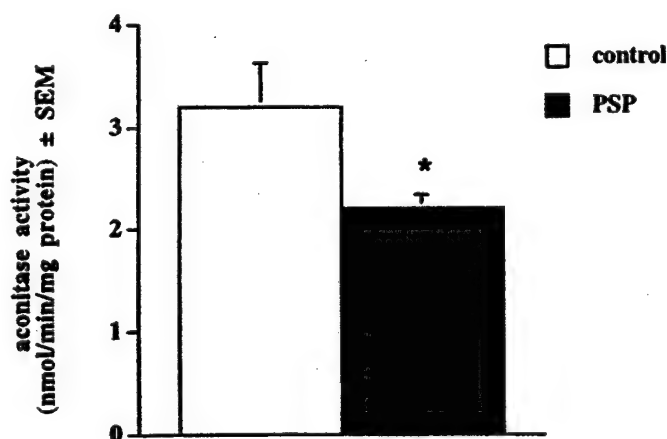


FIG. 1. Means and standard error of the means of aconitase activity in PSP ($n = 14$) and control ($n = 8$) cybrids. Aconitase activities are given as nmol/min/mg protein. *Significantly different from control cybrids ($P < 0.05$).

TABLE 1

Parameters of Mitochondrial Function in PSP and Control Cybrids

Cybrid	<i>n</i>	ATP levels	O ₂ consumption
Control	8	20.3 \pm 1.6	13.2 \pm 0.6
PSP	14	16.9 \pm 1.0*	10.9 \pm 0.5*

Note. Means and standard error of the means of cellular ATP levels and oxygen consumption in control and PSP cybrids. ATP levels are given as nmol/mg protein. Oxygen consumption levels are given as nmol O₂/min.

* Significantly different from control cybrids ($P < 0.05$).

Results from analysis of aconitase activity are presented in Fig. 1. A statistically significant decrease in aconitase activity (-31.25%; $P = 0.02$) was observed in PSP cybrids (2.2 ± 0.1 nmol/min/mg protein, $n = 14$) as compared to control cybrids (3.2 ± 0.4 nmol/min/mg protein, $n = 8$). Parameters of mitochondrial function are shown in Table 1. Both cellular ATP levels and oxygen consumption were significantly decreased (-17%; $P = 0.01$) in PSP cybrids as compared to control cybrids.

Our evidence for mitochondrial impairment in PSP cybrids is not only consistent with a recent study showing significant decreases in complex I activity in PSP cybrids (20) but further establishes the notion that mitochondrial dysfunction plays a contributory role in the pathogenesis of PSP. The changes in parameters of mitochondrial function in the PSP cybrids suggest defects of the respiratory chain of PSP platelets, presumably due to mtDNA abnormalities since all cell lines possess the same nuclear background and were treated identically while in culture. Impaired respiratory chain function results in a decrease in energy production (i.e., \downarrow ATP levels) and leads to a number of deleterious consequences, including increased free radical production and oxidative damage to proteins, membrane lipids, and DNA in several neurodegenerative diseases (for review, see 3). The mtDNA is particularly susceptible to free radical attack due to a lack of protective histones, limited DNA repair capabilities, and proximity to the source of most free radicals in the cell—the electron transport chain. Thus, whether these functional abnormalities observed in the PSP cybrids result from inherited or acquired mtDNA mutations remains to be determined. Future experiments that sequence mtDNA from these cells will help to identify pathogenic mutations that could account for these functional mitochondrial abnormalities.

The roles of mitochondrial dysfunction and oxidative stress in the etiology of other neurodegenerative diseases are widely recognized (3). For example, decreased complex I activity in PD, complex II and aconitase activity in HD, and complex IV and KGDHC activity in AD are well-established. Interestingly, a

shared feature of a number of these enzymes is their sensitivity to oxidation. For instance, low concentrations of H_2O_2 as well as nitric oxide inactivate KGDHC while other enzymes remain unaffected (12, 15). The activity of aconitase, an iron-sulfur protein that catalyzes the first two steps of the citric acid cycle, has been shown to be sensitive to nitric oxide/superoxide/peroxynitrite-mediated damage (4, 5, 7, 11, 13) and reduced in Huntington's disease and Friedreich ataxia (14, 21). Our previous studies showing increased tissue malondialdehyde levels and reduced KGDHC activity in postmortem PSP brain (1, 2) together with the present aconitase data and a study showing increased iNOS immunoreactivity in PSP brain (9) suggest that increased free radical production and mitochondrial dysfunction may play critical roles in the pathogenesis of PSP. Future studies will be required to investigate if oxidative damage to these proteins and to mtDNA alters their function, thereby leading to impaired cellular functioning and ultimately cell death.

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Transgenic ALS Mice Show Increased Vulnerability to the Mitochondrial Toxins MPTP and 3-Nitropropionic Acid

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The pathogenesis of neurodegenerative diseases may involve a genetic predisposition acting in concert with environmental toxins. To test this hypothesis we examined whether transgenic mice with the G93A mutation in Cu,Zn superoxide dismutase show increased vulnerability to either 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 3-nitropropionic acid (3-NP). Compared to littermate controls G93A transgenic mice showed a greater loss of striatal dopamine, DOPAC, and HVA at 50, 70, and 120 days of age following administration of MPTP; however, cell loss in the substantia nigra was not greater. The G93A transgenic mice showed significantly increased vulnerability to striatal lesions produced by 3-NP compared with littermate controls at 120 days of age. The finding that G93A mice show increased vulnerability to mitochondrial toxins further implicates mitochondrial dysfunction in the pathogenesis of neuronal death in these mice. The findings support the hypothesis that a genetic defect can increase susceptibility to environmental toxins and that this may play a role in the pathogenesis of neurodegenerative diseases. © 2001 Academic Press

Key Words: mitochondria; Parkinson's disease; amyotrophic lateral sclerosis; oxidative damage; 3-nitropropionic acid; MPTP.

INTRODUCTION

The pathogenesis of cell death in neurodegenerative diseases remains a challenge for neurobiology despite rapid advances in the molecular genetics of these diseases. Although genetic factors play a major role in a number of neurodegenerative diseases they do not account for all of the phenotypic variation. For instance in Huntington's disease the number of CAG repeats is estimated to account for 60% of the variance in age of onset, suggesting that additional genetic or environmental modifiers may play an important role (22). A

hypothesis which would be consistent with the observations is that disease pathogenesis may be due to a genetic predisposition acting in concert with an environmental toxin. There is precedence for this with a ribosomal RNA mutation in the mitochondrial genome which sensitizes individuals to deafness induced by exposure to aminoglycoside antibiotics (16, 28). There is also evidence that prothrombin gene mutations predispose to cerebral vein thrombosis (27). In Parkinson's disease (PD) there is considerable evidence for both genetic and environmental factors (26, 33). Furthermore hybrid cell lines created from platelet mitochondria from PD patients show a complex I defect, and they show increased vulnerability to 1-methyl-4-phenylpyridinium (MPP⁺) (35).

A major advance in the understanding of the pathogenesis of amyotrophic lateral sclerosis (ALS) was the finding that mutations in Cu,Zn superoxide dismutase (SOD1) are associated with familial ALS (31). The means by which these mutations cause disease appear to be a gain of a deleterious function of the mutant enzyme, since a knockout of SOD1 in transgenic mice does not result in motor neuron death (29). Overexpression of mutant SOD1 results in death of motor neurons in transgenic mice, whereas comparable overexpression of wild-type SOD1 results in no loss of motor neurons (21, 30, 39). The gain of function of the mutant SOD1 protein may be related to abnormal protein aggregation or to toxicity of Cu²⁺ or Zn²⁺ (10). Substantial evidence indicates that the mutations destabilize the protein backbone of SOD and allow the active site Cu²⁺ to become more indiscriminate with regard to substrates (8, 15). The mutations in SOD1 increase reactivity with H₂O₂ or with peroxynitrite *in vitro* and *in vivo* (4, 7, 38, 40, 41). This can result in increased production of hydroxyl radicals and/or nitronium ions which can nitrate tyrosines (14). In transgenic mice overexpressing the G93A SOD1 mutation there is evidence for oxidative damage to both lipids

and protein (17), and there are increased levels of 3-nitrotyrosine in transgenic mice with both the G37R and the G93A SOD1 mutations (12, 17).

There is also evidence that there may be mitochondrial dysfunction in transgenic mice with SOD1 mutations. Mitochondrial vacuolization and swelling are characteristic pathologic features of both the G37R and the G93A transgenic ALS mice (21, 39). Recent studies showed that mitochondrial vacuolization markedly increases prior to a rapid phase of deterioration in muscle strength in G93A mice (23). We found increased mitochondrial complex I activity in cerebral cortex of both familial ALS patients with the A4V SOD1 mutation as well as in the G93A mice (11). Expression of SOD1 with the G93A mutation *in vitro* results in loss of mitochondrial membrane potential as well as increases in cytosolic calcium levels (13).

We therefore examined whether transgenic mice with the G93A SOD1 mutation show increased vulnerability to environmental toxins associated with human illness. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a mitochondrial toxin which produces a parkinsonian syndrome in man (6). Similarly 3-nitropropionic acid (3-NP) is an irreversible inhibitor of succinate dehydrogenase that produces striatal pathology in man. We examined whether transgenic mice with the G93A mutation in SOD1 associated with familial ALS (FALS) show increased vulnerability to the toxic effects of MPTP and 3-NP.

MATERIALS AND METHODS

Hemizygote male (ages 2–4 months) G93A mice [B6SJL-TgN (SOD1-G93A)1 Gur; The Jackson Laboratory, Bar Harbor, ME] were bred with littermate controls to produce transgenic and nontransgenic mice. Mice were genotyped using a polymerase chain reaction assay with primers which recognize human SOD. As adults the transgenic G93A mice develop progressive severe paralysis with death at a mean of 145 days of age (21).

We examined two dosing regimens of MPTP administration in littermate control and G93A transgenic ALS mice at 50, 70, and 120 days of age since we wished to determine whether the G93A mice show increased vulnerability. We used a lower dose at 70 and 120 days of age since MPTP toxicity is age-dependent. MPTP (Research Biochemicals, Wayland, MA) was dissolved in 0.1 ml of phosphate-buffered saline (PBS) and the pH was adjusted to 7.4. At 50 days of age either MPTP or PBS was administered at a dose of 15 mg/kg ip q 2 h \times 5. Fifteen to 20 animals were examined in each group. At 70 and 120 days of age we administered either PBS or MPTP at a dose of 15 mg/kg ip \times 3. Eight animals were examined in each group. Animals were sacrificed at 1 week and the striata were rapidly dissected and placed in chilled 0.1 M

perchloric acid. Tissue was subsequently sonicated and centrifuged and the supernatants were utilized to measure dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) by high-performance liquid chromatography with electrochemical detection (3).

Histologic evaluation was performed on 50-, 70-, and 120-day-old littermate control and G93A transgenic ALS mice (12–18 animals in each group). These mice were deeply anesthetized and then transcardially perfused with 4% buffered paraformaldehyde. The brains were removed, postfixed with the perfusate for 2 h, and cryoprotected in a graded series of 10 and 20% glycerol/2% DMSO solution. The brain tissue specimens were serially sectioned at 50- μ m into a six-well storage container using a frozen-section microtome. All tissue sections from individual storage wells were subsequently stained for Nissl substance and immunohistochemically for tyrosine hydroxylase (TH) (TH antisera, 1:1000 dilution; Eugene Tech International) and dopamine transporter (DAT) (DAT antisera, 1:500 dilution; Chemicon International, Inc.) (15). DAT labels neurons of the substantia nigra pars compacta (5).

All midbrain sections through both the left and the right substantia nigra from the bregma levels \bar{n} 3.08 to \bar{n} 3.16 mm and intraaural levels 0.72 to 0.64 mm in each of the G93A, 1% creatine-fed G93A, and nontransgenic littermate mice were analyzed by microscopic videocapture (18). Videomaging allows for greatly increased magnification and color differentiation capabilities in determining identification of neurons. Neuronal counts of Nissl-, TH-, and DAT-positive neurons within the entire section of the substantia nigra pars compacta and reticulata were calculated using Neurolucida (Microbrightfield) image analysis software. The dissector counting technique was employed, in which all neurons were counted in an unbiased selection of serial sections in a defined volume of the substantia nigra (34, 37).

To determine whether MPTP uptake or metabolism was altered in G93A mice we administered a dose of MPTP of 15 mg/kg q 2 h \times 5 to eight G93A mice and eight littermate control mice at 50 days of age and doses of MPTP of 15 mg/kg q 2 h \times 3 at 70 days of age. Mice were sacrificed at 1 h and the striata dissected. MPP⁺ levels were quantified by HPLC with UV detection at 295 nm. Samples were sonicated in 0.1 M perchloric acid and an aliquot of supernatant was injected onto a Brownlee Aquapore X03-224 cation exchange column and eluted with 90% 0.1 M acetic acid and 75 mM triethylamine HCl (pH 2.3) with 10% acetonitrile.

3-NP was obtained from Sigma (St. Louis, MO) and dissolved in phosphate-buffered saline. 3-NP was administered to 19 G93A transgenic mice and 9 littermate controls at a dose of 50 mg/kg ip b.i.d. for 11 doses starting at 120 days of age. The mice were then deeply anesthetized and transcardially perfused with saline

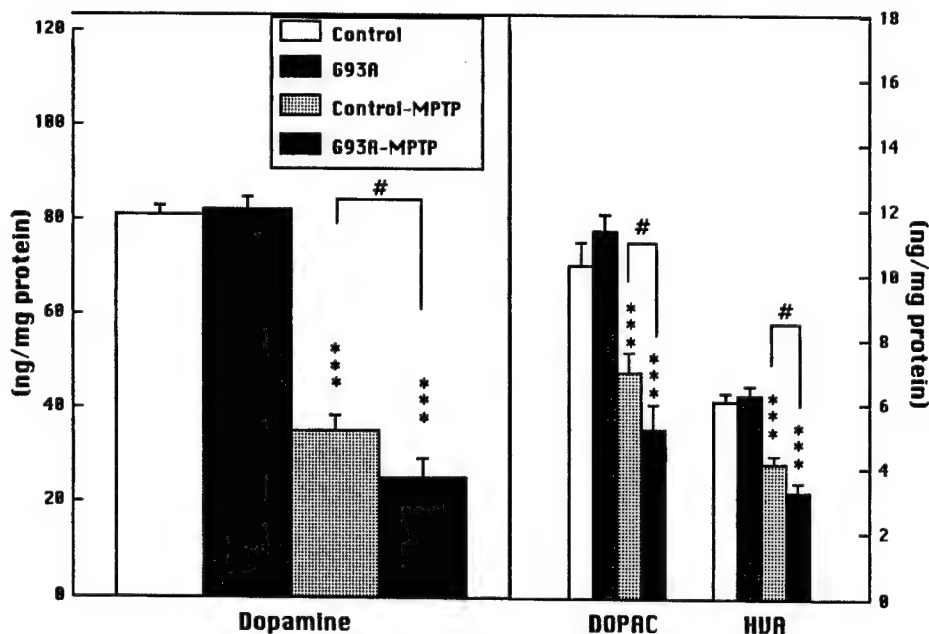


FIG. 1. The effects of MPTP 15 mg/kg \times 5 on striatal dopamine, DOPAC, and HVA in littermate control and G93A mice at 50 days of age. *** $P < 0.001$ compared with control or G93A without MPTP. # $P < 0.05$ compared between control and G93A mice treated with MPTP. $n = 15$ –20/group.

followed by 4% buffered paraformaldehyde 3 h after the last dose (2).

The brains were processed as above and stained for Nissl (cresyl violet). Analysis of lesion volumes was computed from serial sections through the striata of littermate control and G93A transgenic mice using the methods described above.

Statistical comparisons were made by two-way analysis of variance followed by the Tukey post hoc test to compare group means. Neurochemical measurements and neuronal counts are expressed as the means \pm standard errors of the mean. All animals used in these procedures were in strict compliance with the NIH Guide for the Care and Use of Laboratory animals and were approved by all local Animal Care Committees.

RESULTS

At 50 days of age there were no significant differences in dopamine, DOPAC, or HVA in littermate controls compared with G93A transgenic mice. Following administration of MPTP there were significant decreases in dopamine, DOPAC, and HVA in both littermate controls and G93A transgenic mice (Fig. 1). The decreases in dopamine, DOPAC, and HVA, however, were significantly greater ($P < 0.05$) in the G93A transgenic mice than in the littermate controls.

At 70 days of age there were also no significant differences in dopamine, DOPAC, or HVA in littermate controls compared with G93A transgenic mice (Fig. 2). Following administration of MPTP there were significant decreases in dopamine in the G93A mice but not

in the control mice. Similarly both DOPAC and HVA were significantly decreased in G93A but not in littermate control mice. The depletions of dopamine and HVA were significantly greater in the G93A mice compared to littermate control mice.

The measurements of dopamine, DOPAC, and HVA showed a small significant decrease in dopamine, but no changes in DOPAC or HVA between littermate controls and G93A transgenic mice following administration of PBS at 120 days of age (Fig. 3). Following administration of MPTP there was a significant decrease in dopamine concentrations in both the littermate control mice and the G93A transgenic mice. The dopamine depletion in the littermate control mice was 32%, whereas in the G93A mice it was 48%. This was significantly greater than the depletion in the littermate controls, $P < 0.01$. In addition there was a significant depletion of DOPAC in the G93A MPTP-treated mice; however, a small depletion in the littermate controls was not significant. The depletion of DOPAC and HVA following MPTP in G93A mice was significantly greater than that in littermate controls ($P < 0.05$). We examined MPP⁺ levels to be certain that there was no alteration in MPTP uptake or metabolism. MPP⁺ levels were not significantly different in littermate control and G93A mice at 50 days of age (80.9 ± 5.5 versus 81.0 ± 3.5 ng/mg protein) or at 70 days of age (73.6 ± 9.5 versus 69.6 ± 13.5 ng/mg protein).

Neuronal counts of Nissl-stained and TH- and DAT-immunostained neurons showed no cell loss in G93A mice compared to littermate controls at 50 days of age

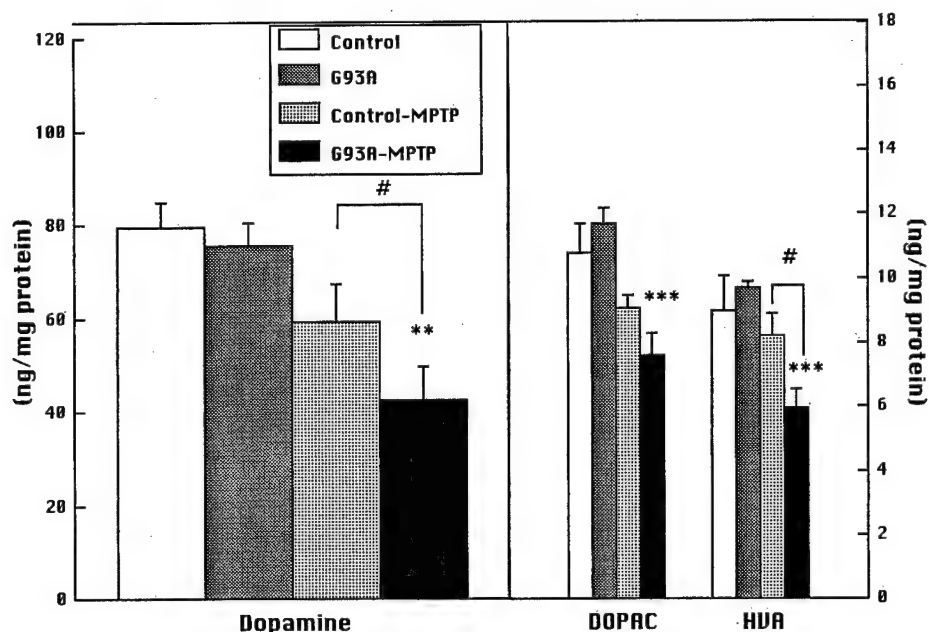


FIG. 2. The effects of MPTP 15 mg/kg \times 3 on striatal dopamine, DOPAC, and HVA in littermate control and G93A mice at 70 days of age, $**P < 0.01$, $***P < 0.001$ in MPTP-treated G93A mice compared with phosphate-buffered saline-treated G93A mice. $\#P < 0.05$ compared between control and G93A mice treated with MPTP. $n = 8/\text{group}$.

(Table 1). In comparison, the loss of substantia nigra neurons in both MPTP-treated G93A and littermate control mice was significant (Table 1). While the reduction of nigral neurons was greater in the G93A mice it was not significantly greater than the littermate control group treated with MPTP.

As others and we previously found, there was a significant loss of substantia nigra neurons in the G93A mice at 120 days in comparison to their littermate transgene-negative controls (Table 2). In addition MPTP treatment resulted in a marked neuronal loss in both G93A and control mice. The susceptibility to

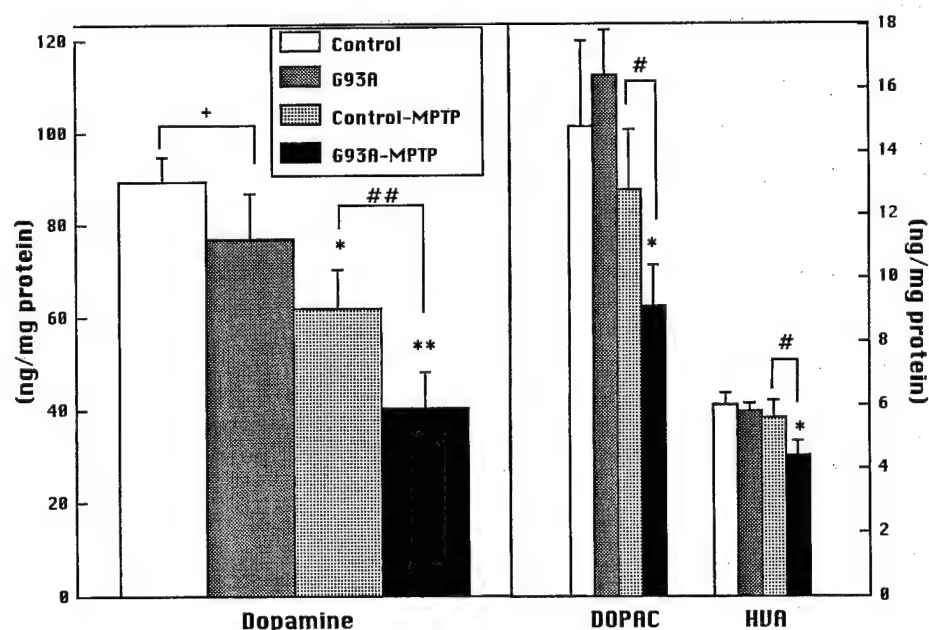


FIG. 3. The effects of MPTP 15 mg/kg \times 3 on striatal dopamine, DOPAC, and HVA in littermate control and G93A mice at 120 days of age. $*P < 0.05$ in control compared to G93A mice. $*P < 0.05$, $**P < 0.01$ compared with control or with G93A without MPTP. $\#P < 0.05$, $\#\#P < 0.01$, compared between control and G93A mice treated with MPTP. $n = 8/\text{group}$.

TABLE 1

Nissl, Tyrosine Hydroxylase (TH), and Dopamine Transporter Protein (DAT) Neuronal Counts in the Substantia Nigra of 50-Day-Old ALS Transgenic and Littermate Control Mice Treated with MPTP

	Nissl	TH	DAT
Littermate control mice	153 ± 6.3	124 ± 4.7	117 ± 5.3
G93A mice	143 ± 7.6	117 ± 4.8	110 ± 5.1
Littermate control mice, MPTP-treated	116 ± 16.1*	97 ± 12.9*	92 ± 11.2*
G93A mice, MPTP-treated	106 ± 17.1**	89 ± 14.6**	83 ± 13.8*

Note. Nissl, tyrosine hydroxylase, and dopamine transporter-positive neurons within the substantia nigra were counted in 12 brains from each group. Data are means ± SEM.

* $P < 0.05$, ** $P < 0.01$ compared with PBS-treated controls. The extent of cell loss in the G93A mice was not significantly greater in the ALS mice compared to controls.

MPTP was greater in 120-day-old G93A mice than that observed in the 50-day-old G93A mice ($P \leq 0.01$); however, neuronal loss was not significantly greater in the G93A mice than the wild-type mice.

In both littermate control and G93A transgenic mice treated with 3-NP, histologic examination identified bilateral ovoid areas of staining pallor in the striata (Fig. 4). These zones represented lesions in which there was marked neuronal loss and astrogliosis. The lesion volumes in the striatum were significantly greater in the G93A transgenic mice than those present in littermate controls (G93A transgenic mice, $10.7 \pm 4.3 \text{ mm}^3$; littermate control mice, $1.1 \pm 0.3 \text{ mm}^3$; $P \leq 0.001$).

DISCUSSION

The G93A SOD1 mutation may cause both mitochondrial dysfunction and oxidative damage that may result in increased vulnerability to mitochondrial toxins. We therefore examined whether transgenic mice with the G93A SOD1 mutation show increased vulnerability to MPTP and 3-NP, which are environmental toxins which are known to cause human illness. MPTP is a selective dopaminergic neurotoxin which produces a parkinsonian syndrome both in man and in nonhuman primates (6). The mechanism involves conversion of MPTP to MPP^+ by monoamine oxidase B, followed by uptake of MPP^+ by the dopamine transporter, accumulation in mitochondria, and inhibition of complex I of the electron transport chain (19, 36).

A complicating factor in the interpretation of MPTP toxicity is the finding of a significant loss of tyrosine hydroxylase-positive neurons in the substantia nigra in G93A FALS mice. A 26% loss of these neurons was seen at end-stage illness and this was accompanied by

an 18% loss of dopamine in the striatum compared with littermate controls (24). We also found a 25% loss of substantia nigra tyrosine hydroxylase neurons in G93A mice at 120 days of age and a significant 14% decrease in dopamine levels at this time point. As reported previously this cell loss is not seen in 1-month-old mice and we did not see either cell loss or depletion of dopamine levels at 50 or 70 days of age.

We therefore examined the susceptibility of transgenic G93A mice to MPTP toxicity at 50, 70, and 120 days of age. Compared to littermate controls the extent of depletion of dopamine, DOPAC, and HVA was significantly greater in the G93A mice at 50, 70, and 120 days of age. There was also a trend toward a greater loss of substantia nigra tyrosine hydroxylase neurons in the G93A compared with control mice; however, the difference was not significant at either 50 or 120 days of age. Using a milder dosing regimen we also observed significantly greater loss of dopamine, DOPAC, and HVA in G93A mice at 120 days of age. This result is confounded by the 14% decrease in dopamine levels we found at baseline; however, the decrease following MPTP was still significantly greater than that seen in controls. Similar findings were recently reported in mice with the G86R SOD1 mutation (20).

We also examined the effects of 3-NP in the G93A mice. 3-NP is an irreversible inhibitor of succinate dehydrogenase which causes striatal pathology in man (25). Following systemic administration of 3-NP it results in selective striatal lesions which closely replicate the neurochemical and histopathologic features of HD (1, 9). The mechanism of cell death involves both mitochondrial dysfunction and oxidative damage mediated by peroxynitrite (32). In the present experiments we found that mice with the G93A mutation show increased vulnerability to striatal lesions produced by

TABLE 2

Nissl, Tyrosine Hydroxylase (TH), and Dopamine Transporter Protein (DAT) Neuronal Counts in the Substantia Nigra of 120-Day-Old ALS Transgenic and Littermate Control Mice Treated with MPTP

	Nissl	TH	DAT
Littermate control mice	149 ± 5.7	123 ± 5.3	111 ± 4.6
G93A mice	121 ± 14.1	84 ± 6.2	78 ± 7.1
Littermate control mice, MPTP-treated	72 ± 15.8*	57 ± 14.1**	52 ± 10.7**
G93A mice, MPTP-treated	45 ± 13.2**	38 ± 14.6**	34 ± 12.8**

Note. Nissl, tyrosine hydroxylase, and dopamine transporter-positive neurons within the substantia nigra were counted in 12 brains from each group. Data are means ± SEM.

* $P < 0.01$, ** $P < 0.001$ compared with controls. The cell loss in the ALS mice was not greater than that in the controls.

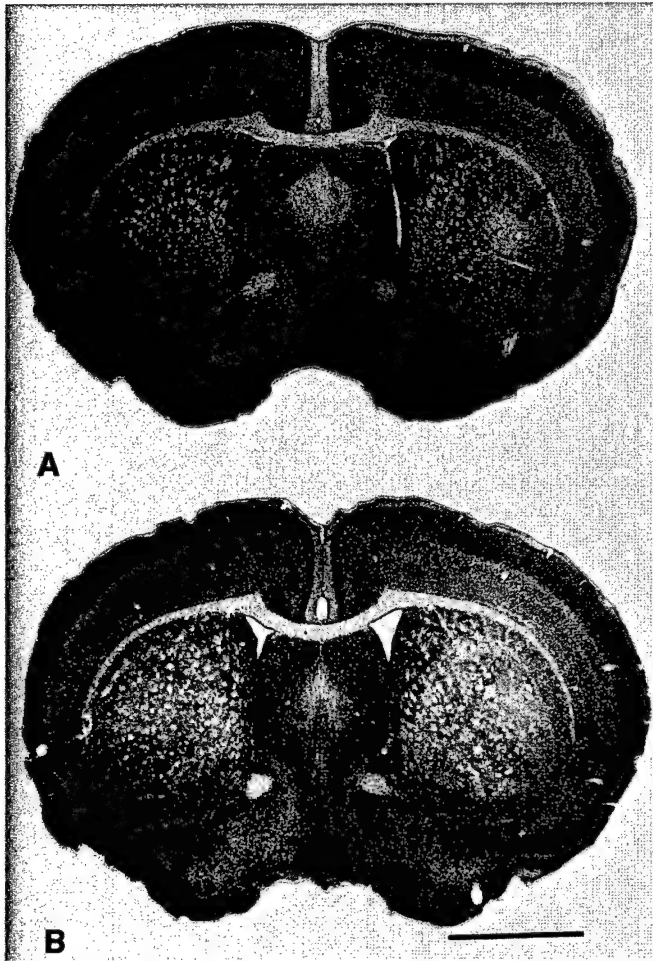


FIG. 4. Photomicrographs of coronal brain-sections at the level of the anterior commissure from 3-NP-treated G93A transgenic-negative littermate control (A) and G93A transgene-positive (B) mice. Significantly greater bilateral striatal lesions are observed in the G93A transgene-positive animal (B) in contrast to the littermate control (A) (arrows). Magnification bar, 2 mm.

3-NP compared to littermate controls. We recently used *in vivo* microdialysis to show that G93A mice show increased production of "hydroxyl-like" radicals at baseline and show a further increase in response to 3-NP, which is greater than that seen in controls (7).

The present findings therefore implicate both mitochondrial dysfunction and oxidative damage in the G93A mice. They show that lesions produced by two mitochondrial toxins are exacerbated in G93A mice. If these mice have both mitochondrial dysfunction and increased oxidative damage at baseline, one would expect that they may be more susceptible to toxins which produce further impairment of mitochondrial function and oxidative stress. These findings are consistent with the hypothesis that genetic defects in man may act together with environmental toxins in the pathogenesis of neurodegenerative diseases. There is precedence for this in Parkinson's disease, in which there is

evidence for mitochondrial dysfunction affecting complex I activity as well as evidence for environmental risk factors (33, 35). A mild defect in mitochondrial function or a defect in free radical scavenging may therefore be able to act in concert with environmental mitochondrial toxins in the pathogenesis of human neurodegenerative diseases.

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Potential for Creatine and Other Therapies Targeting Cellular Energy Dysfunction in Neurological Disorders

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Substantial evidence indicates that bioenergetic dysfunction plays either a primary or secondary role in the pathophysiology of cell death in neurodegenerative and neuromuscular disorders, and even in normal aging. Agents that ameliorate bioenergetic defects may therefore be useful in therapy. Creatine, which increases muscle and brain phosphocreatine concentrations, and may inhibit the activation of the mitochondrial permeability transition, protects against neuronal degeneration in transgenic murine models of amyotrophic lateral sclerosis and Huntington's disease and in chemically mediated neurotoxicity. Initial studies of creatine use in humans appear promising; however, further long-term, well-designed trials are needed. Coenzyme Q₁₀, *Ginkgo biloba*, nicotinamide, riboflavin, carnitine, lipoic acid, and dichloroacetate are other agents which may have beneficial effects on energy metabolism, but the preclinical and clinical evidence for efficacy in neurological diseases remains limited. These compounds are widely used as dietary supplements; however, they must be subjected to rigorous evaluation through randomized, double-blinded trials to establish efficacy, cost-effectiveness and safety in neurological disorders.

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There are a number of similarities in the fundamental biochemical processes involved in the pathogenesis and progression of otherwise different neurological diseases. The concepts of excitotoxicity, oxidative stress, energy depletion, and mitochondrial dysfunction have been implicated in such disorders as Huntington's disease,^{1,2} Parkinson's disease,^{3,4} amyotrophic lateral sclerosis (ALS),^{3,5} and mitochondrial cytopathies.⁶⁻⁸ Although these biochemical processes may be directly or indirectly involved in the pathogenesis of a given disorder, these interrelated processes converge in "final common pathways" of either necrosis or apoptosis.

Mitochondria are critical organelles in the regulation of cellular energy status (aerobic metabolism). However, they are also directly or indirectly involved in excitotoxicity,⁹ the generation of free radicals,⁷ calcium buffering,¹⁰ and apoptosis.¹¹ Oxidative stress may also be a factor in the generation of mitochondrial deoxyribonucleic acid (DNA) mutations in aging and Alzheimer disease (AD).^{12,13} Mitochondrial mutations, particularly at Complexes I and III, can lead to further generation of free radicals^{7,14,15} and contribute to a positive feedback cycle. Because all of these processes converge upon similar cellular functions, they provide

many potential targets for the therapy of neurological disease. A summary of some of the processes relevant to neurological disorders is presented in Figure 1.

Although much effort has been spent on the development and testing of drugs that target receptor mediated processes such as excitotoxicity (ie, riluzole for ALS),¹⁶ the pharmaceutical and nutraceutical development and testing of antioxidant and energy yielding compounds has been less vigorous. Compounds should first be tested for efficacy and safety in cell culture and animal models and then tested in human disease provided that the sensitivity/specificity and reliability of the outcome measures are known. The outcome measure should be designed to evaluate a biological process that the experimental compound is meant to alter. For example, a study of an antioxidant in mitochondrial disease is not likely to show a beneficial effect on short-term strength, whereas it may show an effect upon lipid peroxidation.

This review focuses on the potential therapeutic efficacy of compounds that can enhance cellular energy transduction (ie, creatine monohydrate, nicotinamide) and/or function as antioxidants (ie, *Ginkgo biloba*, lipoic acid). These two cellular functions are related, and

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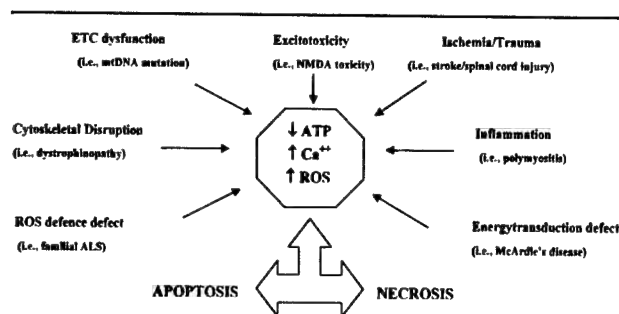


Fig. Some neurological pathologies that may be involved in neuronal/muscle damage. ROS = reactive oxygen species; ETC = electron transport chain; ATP = adenosine triphosphate; ALS = amyotrophic lateral sclerosis; mtDNA = mitochondrial DNA; NMDA = N-methyl-D-aspartate.

a single compound may directly target one process and have a secondary effect upon the other process.¹⁷ A summary of some of the proposed mechanisms of action of these agents is found in Table 1. Creatine monohydrate will be the major focus of the review owing to a recent increase in the number of publications that have examined the biological role of creatine and the creatine kinase system in neurology,^{6,17-25} and because the results of several clinical trials in neurological disease have recently been published.²⁶⁻³⁰ Although some of the fundamental cellular processes involved in normal aging may be similar to those in neurological disorders (eg, oxidative stress, motor unit loss, muscle atrophy), we shall limit the current review to neurological disorders per se. The reader may refer to a recent review of the potential role for creatine monohydrate in aging.³¹

Creatine Monohydrate

Creatine Structure, Metabolism, and Role in the Cell

Creatine is a guanidino compound found in meat-containing products and produced endogenously by the liver, kidneys, and pancreas.³² The production of creatine requires the amino acids arginine and glycine as well as methionine (provision of a methyl group). The rate limiting enzyme, L-arginine:glycine amidinotransferase (transamidinase), results in the production

of guanidinoacetate, which in turn is methylated by S-adenosyl-methionine [catalyzed by guanidinoacetate-methyltransferase (GAMT)] to produce creatine.³³ The importance of creatine to neurological functioning can be demonstrated by the finding of developmental delay, hypotonia, extrapyramidal movement disorders, and seizures in patients with an inborn error of metabolism in creatine biosynthesis (GAMT deficiency).^{34,35} In two cases, many of the neurological impairments were reversed after creatine monohydrate supplementation.^{36,37}

Creatine is taken up into brain, cardiac, and skeletal muscle by a sodium-dependent transporter that has been cloned and sequenced.³⁸ Inward creatine transport is enhanced in the presence of insulin,³⁹ whereas transport is attenuated by a high exogenous creatine concentration.⁴⁰ The effect of exogenous creatine upon creatine transport may be due to a downregulation of creatine transporters.⁴¹ However, in the latter study the creatine dose given to the rats was several times higher than a usual human dose,⁴¹ and we have recently shown that the creatine transporter is not downregulated in humans after two months of high physiological dose of creatine monohydrate (10 g/day).⁴² The concentration of phosphocreatine (PCr) in resting skeletal muscle is lower in patients with muscular dystrophy/congenital myopathies,²⁴ inflammatory myopathies,²⁴ Huntington's disease,^{43,44} Friedrich's ataxia,⁴⁵ and mitochondrial cytopathies,⁴⁶ and with normal aging.⁴⁷ Brain PCr concentration is lower in patients with mitochondrial cytopathy,⁴⁸ cerebral ischemia,⁴⁹ and bipolar affective disorders,⁵⁰ and in animal models of cerebral ischemia.⁵¹ Therefore, the potential exists for creatine to be a therapeutic replacement strategy in neurological disease.

Within the cell, creatine exists as both free Cr and PCr, which together make up the total creatine pool (TCr). In tissues such as muscle and brain, PCr functions as a temporal energy buffer in which ADP is rephosphorylated to adenosine triphosphate (ATP) during periods of high energy demand ($\text{PCr} + \text{ADP} + \text{H}^+ \leftrightarrow \text{Cr} + \text{ATP}$). This phosphoryl group transfer is catalyzed by cytosolic creatine kinase (CK). Muscle PCr

Table 1. Agents with Effects on Brain Bioenergetics and Proposed Mechanisms of Action

Agent	Proposed Mechanism of Action	Reference
Creatine	↑ PCr concentration, inhibits the MPT	19, 25, 62, 76
CoenzymeQ ₁₀	Cofactor of Complex I, II, III, and antioxidant	112, 114, 117
Ginkgo biloba	Antioxidant and preserves mitochondrial function	135, 139, 140
Nicotinamide	Precursor of NADH, inhibitor of poly-ADP-ribose polymerase	144, 148
Riboflavin	Precursor of FAD, cofactor of Complex I and Complex II	151, 154
Carnitine	Facilitates long-chain fatty acid entry into the mitochondria	157, 164
Lipoic acid	Coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, antioxidant	166, 167, 170
Dichloroacetate	Stimulates pyruvate dehydrogenase	177, 179

breakdown provides for ~50% of the energy during the first 10 seconds of muscle contraction.⁵² Postexercise PCr recovery requires oxygen, and the majority of PCr is resynthesized within 2 minutes in healthy humans.^{53,54} The recovery of PCr concentration is markedly impaired in patients with aerobic energy defects (ie, mitochondrial cytopathies).^{46,55} Strategies that lower the intramuscular concentration of Cr can slow contraction speed⁵⁶ and decrease peak force.¹⁰ This temporal energy buffering system is the main target of dietary CM loading strategies to enhance athletic performance (for review see Ref 16). In addition to its role in skeletal muscle, the Cr/PCr system has an important role in mature and developing brain metabolism.⁵⁷

The Cr/PCr system also functions as a spatial energy buffer between the cytosol and mitochondria using a unique mitochondrial CK isoform (mtCK).^{23,58} The mtCK isoform exists in the intermembranous space of the mitochondria in an octameric and dimeric form.⁵⁹ The octameric form facilitates the functional coupling between porin (outer mitochondrial membrane) and the adenine nucleotide translocase (inner mitochondrial membrane).⁵⁹ Together, porin and the adenine nucleotide translocase form components of the mitochondrial permeability transition (MPT), whose opening is inhibited when mtCK is in the octameric form.⁶⁰ The mtCK isoform dimerizes in the presence of free radicals such as peroxynitrite,⁶¹ which can negatively affect calcium handling, open the MPT, and impair State 3 and State 4 respiration.⁶² Both Cr and PCr can attenuate the peroxynitrite mediated mtCK inactivation and dimerization⁶¹ and opening of the MPT.^{25,62}

Creatine supplementation attenuates the accumulation of markers of oxidative stress in animal models of Huntington's disease¹⁷ and ALS.⁶³ Creatine per se would not be expected to function as an acceptor of an unpaired electron, but likely functions as an antioxidant through an enhancement of energy transduction (indirect antioxidant function). The antioxidant function of creatine would attenuate the inactivation of mtCK and opening of the MPT.^{22,62,64} Along these lines, creatine also increases glutamate uptake into synaptic vesicles,⁶⁵ which could potentially attenuate neuronal excitotoxicity.

Another negative consequence of dimeric mtCK is that the molecule can crystallize and form the paracrystalline inclusions that are seen in some mitochondrial cytopathies⁶ and after creatine depletion in skeletal muscle.⁶⁶ The reversal of paracrystalline inclusions has been demonstrated in rats after creatine supplementation,⁶⁶ and we recently demonstrated the reversal of paracrystalline inclusions in a patient with a missense mutation in the cytochrome b gene.⁶⁷

The Cr/PCr system may also function as a stimulator of myofibrillar protein synthesis,^{68,69} which may partially explain the observed increase in lean body

mass seen in studies of creatine supplementation in young healthy human subjects.^{70,71} Creatine promotes an increase in satellite cell activation during compensatory hypertrophy in rats.⁷² There is also a close functional coupling between the sarcoplasmic Ca^{2+} ATPase and the CK system.⁷³ This functional coupling may explain the faster skeletal muscle relaxation time observed after creatine loading in humans,⁷⁴ and the impairment in skeletal muscle excitation/contraction shown using a double cytosolic and mtCK knockout mouse.¹⁰ Finally, the enhancement of Ca^{2+} uptake may explain the reduction in cytosolic Ca^{2+} accumulation and enhanced survival of muscular dystrophy (*mdx*) myotubes in the presence of creatine.²²

As outlined above, there are many potential beneficial effects from creatine supplementation that would be predicted to occur in neurological disorders in which oxidative stress, muscle atrophy, muscle fatigue, apoptosis, and altered Ca^{2+} metabolism play a role.

Dietary Intake/Supplementation

Dietary Cr is found in meat products (eg, 5g of Cr is found in about 1.1 kg of beef), with a typical North American diet providing about 1 g of Cr/day.^{32,75} Exogenous Cr is also available as a supplement in the form of Cr monohydrate (CM). This is a tasteless white powder that is water soluble and commercially available in North America and Europe. The consumption of 20 g of CM for 4 to 5 days (loading) resulted in an increase in total Cr of about 25 mmol/kg dry mass (~17–20%).^{75,76} After dietary loading, muscle total Cr can be maintained by consuming 2 g Cr monohydrate/day and returns to baseline within a month after reinstitution of a normal diet.⁷⁶ The ingestion of 3 g of Cr monohydrate/day for 1 month resulted in muscle concentrations⁷⁶ similar to those seen after the traditional loading described above. People with low muscle total Cr stores have the greatest potential for loading.⁷⁵ Therefore, patients with neurological disorders with low basal Cr concentrations (see above) would likely benefit most from strategies aimed at restoring or enhancing intracellular Cr homeostasis. One study found that the consumption of 20 g CM/day for 4 weeks resulted in a reversible increase in total Cr in the brains of young healthy men [range = 4.7% (gray matter) → 14.6% (thalamus)].⁷⁷ An increase in brain Cr and PCr concentration has also been demonstrated in animal models of ALS,⁶³ Huntington's chorea,^{17,19} and MPTP toxicity.²¹ In healthy human volunteers cerebral PCr was increased approximately 10% by oral creatine administration.⁷⁷ We recently found that the creatine transporter is downregulated in the muscle of patients with muscular dystrophy, inflammatory myopathies, and mitochondrial cytopathies.⁷⁸ Therefore, the dosing requirements for loading in such patients may be higher than for otherwise healthy individuals.

Whole Body Effects of Supplementation in Healthy Humans

Several studies have demonstrated that CM loading resulted in increased power output in single and repeated bouts of high-intensity exercise (fatigue in 10–30 seconds).^{32,70,79–81} Acute loading results in an increase in fat-free mass.⁷¹ Others have reported a greater increase in weight-lifting performance and fat-free mass during several weeks of weight training with CM, compared with placebo.^{82–85}

Safety

It is important that the CM which is used in studies, and which may ultimately be provided to patients, is of the highest chemical purity. Because Cr is endogenously synthesized and consumed in the diet, it is not surprising that there have been few reported side effects in a number of studies in both healthy persons^{75,76,80,83,86,87} and patients.^{27,28,71,79,88} We showed that Cr supplementation (20 g/day \times 5 days) did not affect blood pressure, plasma CK activity, creatinine clearance, or plasma creatinine concentration.⁷¹ There have been two case reports of apparent deterioration in renal function as adverse events during CM supplementation.^{89,90} However, careful prospective studies showed no effect of CM supplementation on renal function^{71,91} for up to 5 years of supplementation.⁹² There was another case report of a stroke in a bodybuilder who was taking MaHuang and creatine,⁹³ however, MaHuang contains ephedra, which is a stimulant associated with death in otherwise healthy humans.⁹⁴

There has been a theoretical concern that long-term CM supplementation could lead to a persistent reduction of Cr synthesis and/or transport.⁹⁵ A reduction in Cr transporters was reported in rats fed 4% dietary CM for 3 to 6 months.⁴¹ This raised the possibility that long-term CM supplementation could lead to a reduction in Cr transporters, reduced efficacy of CM in the long term, and an undershoot in tissue levels upon cessation of supplementation.⁹⁵ However, we have recently found that 2 months of CM supplementation (10g/day) in young male athletes did not result in a downregulation of Cr transporters and total Cr concentrations were maintained.⁴² The difference between the two studies likely relates to the much larger dose of CM given to the rats⁴¹ which would be equivalent to 24 g/day if 4% CM were given to humans on a 2,500 kcal/day diet. There has also been some concern about the long-term effects of this potential suppression of endogenous Cr synthesis, as exogenous CM results in a lower mRNA message for L-arginine:glycine amidinotransferase³³; however, muscle PCr and TCr concentrations do not undershoot after cessation of CM supplementation.^{76,96}

Theoretical Use and Experimental Studies of Creatine in Neurological Disorders

The potential benefits of CM supplementation are likely to be realized in neurological disorders in which cellular energy metabolism is impaired (ie, glycogen storage disease, mitochondrial cytopathy, Huntington's chorea) and/or the disorder results in a reduction in the basal cellular PCr/Cr concentration (ie, muscular dystrophy, inflammatory myopathy, mitochondrial cytopathy). As outlined earlier, many basic cellular functions could be positively enhanced by CM supplementation in such disorders (Fig). Creatine administration protects against glutamate and β -amyloid toxicity in rat hippocampal neurons.¹⁸ Creatine monohydrate supplementation is beneficial in animal models of traumatic brain injury²⁵ and cerebral ischemia.^{51,97} Preincubation of anoxic rat hippocampal slices with CM attenuated the decrease in PCr and ATP content, the reduction in cellular protein synthesis, and neuronal injury.⁹⁸ Cerebral synaptic transmission failure could be delayed threefold when tissue was preincubated in CM, and this was associated with an attenuation of PCr and ATP decreases.⁹⁹ Another study found that brainstem ATP and PCr concentrations were better maintained after ischemia in neonatal pups whose mothers were supplemented with CM.⁹⁷ A recent study found less cortical damage and an attenuation of neuronal apoptosis after traumatic brain injury in rats pretreated with CM.²⁵

We found that oral CM supplementation attenuated striatal lesions produced by 3-nitropropionic (3-NP) acid and malonate, which are irreversible and reversible Complex II inhibitors, respectively, and which model Huntington's disease.¹⁷ There was an attenuation of the ATP and PCr depletion, reduced lactate accumulation, and less oxidative stress (3-nitrotyrosine accumulation).¹⁷ Supplementation with 2% CM attenuated dopamine reduction and neuron loss in the substantia nigra of mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).²¹ Oral administration of 1% CM attenuated striatal lesions produced by *N*-methyl-D-aspartate (NMDA), but not those produced by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainic acid.¹⁰⁰

Creatine monohydrate administration in a transgenic murine model of ALS (G93A) resulted in improved motor performance, a dose-dependent increase in survival, preservation of anterior horn neurons, and reduced oxidative stress.⁶³ A transgenic murine model of Huntington's disease with a large CAG repeat expansion (line R6/2)¹⁰¹ shows both an increase in oxidative stress and mitochondrial dysfunction.¹⁰² Administration of dietary CM to these animals (R6/2) improved motor performance and survival, attenuated the loss of brain and total body weight, and reduced striatal neu-

ron atrophy and the formation of Huntington-positive aggregates.¹⁹

The aforementioned studies show that CM is a useful treatment in animal models of neurodegenerative disorders (Table 2). It appears that 1% and 2% dietary supplementation is the most efficacious dose in rodents, which corresponds to doses that could be given to humans. If one assumes that the average 70-kg male eats a mixed diet providing 2,700 kcal/day, this would amount to about 6 to 12 g/day, respectively (~ 0.086 – 0.172 g/kg/day).

Use of Creatine in Human Neurological Disease

One of the most logical potential uses of CM treatment would be in GAMT deficiency where Cr biosynthesis is reduced.^{35,36,103} In one child with severe neurological defects, CM treatment (4 g/day for 2.5 years) resulted in marked improvement in the extrapyramidal disorder, developmental milestones, magnetic resonance imaging (MRI) abnormalities, and cerebral creatine content in both gray and white matter where levels increased from being nondetectable to about 80% of control values.³⁴ A recent report also found clinical, biochemical, and neuroradiological improvements in a child diagnosed with GAMT deficiency after CM treatment.³⁶ Another report found a generalized brain creatine deficiency in two sisters who presented with mental retardation and severe language delay.¹⁰⁴ They did not have GAMT deficiency or evidence for another synthesis defect, and the authors suggested that the defect was in the Cr transporter.¹⁰⁴ Treatment in these sisters resulted in near complete restoration of brain creatine content and improvement in symptoms.¹⁰⁴

The neuroophthalmological condition, gyrate atrophy, results in a secondary Cr synthesis defect and results in a near-complete Cr deficiency in brain and muscle.⁸⁸ In this inborn error of metabolism, ornithine accumulation inhibits L-arginine:glycine amidinotransferase

(rate-limiting enzyme in creatine biosynthesis),¹⁰⁵ and patients have chorioretinal atrophy, premature cataracts, and Type II muscle fiber atrophy.⁸⁸ One year of low-dose CM supplementation (1.5 g/day) resulted in a 45% increase in Type II muscle fiber diameter, and an attenuation of eye disease progression.⁸⁸ In a 5-year follow-up of 13 gyrate atrophy patients, skeletal muscle Type II fiber atrophy and tubular aggregates were no longer seen; however, the eye findings progressed (thought not to be due to Cr depletion per se).¹⁰⁶ Recently, this group found that brain creatine was reduced in the basal ganglia by $\sim 50\%$, and that CM supplementation at modest doses (1.5–2 g/day) resulted in partial correction of the defect.¹⁰⁷ They also found a correction of reduced skeletal muscle PCr concentration in patients with gyrate atrophy.¹⁰⁸

Human studies have also been completed in the area of mitochondrial cytopathies.^{26,27,109,110} A case report demonstrated improved exercise capacity, reduced headache frequency, and a normalization of electrocardiogram abnormalities after 3 months of CM treatment (10g/day \times 2 weeks \rightarrow 4 g/day \times 2.5 months) in a patient with the MELAS A3243G mutation.¹¹⁰ Another case series found that 3 months of CM supplementation (0.1–0.2 g/kg/day) increased maximal cycle power by 8% to 17% ($p < 0.025$) and submaximal endurance time by 30% to 57% ($p < 0.01$) in four patients with mitochondrial cytopathies (2 = KSS; 1 = NARP; 1 = MELAS).¹⁰⁹ We completed a randomized, double-blind, crossover study of the effects of CM (5 g twice daily \times 14 days \rightarrow 2 g twice daily for 7 days) in seven patients with mitochondrial cytopathy (primarily MELAS).¹⁰⁹ Creatine monohydrate supplementation resulted in a significant increase (11%) in dorsi-flexion power during a 2-minute fatigue test, an increase (19%) in hand grip power during a 60-second fatigue test, and an increase ($\sim 10\%$) in postexercise lactate concentration for both the hand

Table 2. Summary of Creatine Use in Animal Models of Neurological Disease

Study	Model	CM Dose	Outcome (CM vs Placebo)
Matthews et al., 1998 Klivenyi et al., 1999	Malonate/3-NP G93A FALS (mice)	1% and 2% diet 1% and 2% diet	\uparrow [PCr]; \downarrow striatal lesions; \downarrow 3-NT \uparrow survival; \uparrow motor perf.; \downarrow ventral horn neuron loss; \downarrow 3-NT
Matthews et al., 1999	MPTP	2% diet	\downarrow dopamine depletion and neuronal dropout in SN
Malcon et al., 2000	NMDA, kainic acid, AMPA	1% diet	NMDA only; \downarrow striatal lesions
Ferrante et al., 2000	HD transgenic mouse (R6/2)	1%, 2%, and 3% diet	\uparrow survival; \uparrow motor perf.; \downarrow brain wt. loss; \uparrow body wt.; \downarrow striatal ag- gregates; \downarrow striatal neuron atrophy
Sullivan et al., 2000	Head trauma model		\downarrow cortical loss; \uparrow ATP and \uparrow $\Delta\psi_m$, \downarrow ROS and Ca^{2+}

CM = creatine monohydrate; 3-NP = 3-nitropropionic acid; 3-NT = 3-nitrotyrosine; FALS = familial ALS; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SN = substantia nigra; NMDA = *N*-methyl-D-aspartate; AMPA = α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; HD = Huntington's disease; $\Delta\psi_m$ = mitochondrial membrane potential; ROS = reactive oxygen species.

grip and VO_{2peak} tests. There were no effects of CM supplementation upon cycle ergometry VO_{2peak} , activities of daily living, and there were no reported side effects.¹⁰⁹ Similar increases in strength were observed in a larger open trial that contained 17 patients with mitochondrial disease.⁹⁸ However, in a recent randomized, double-blind, crossover trial in 16 patients, no statistically significant benefits were observed after 4 weeks of CM supplementation.²⁶ The difference in outcome between the two randomized trials is likely related to the more severe phenotype of the patients in the smaller study where all patients had lactic acidosis, ragged red fibers, and paracrystalline inclusions in muscle biopsies.²⁷ In the larger study, many of the subjects had chronic progressive external ophthalmoplegia,²⁶ for which we have not found significant reductions in PCr.²⁴ One potentially disturbing observation was the higher postexercise lactate concentration in the trial with the seven more severe patients.²⁷ Because basal concentrations were not elevated, it is likely that the postexercise values were due to the higher workloads achieved.²⁷ Furthermore, in animal models of neurotoxicity, CM reduced basal brain tissue lactate concentrations.^{17,63}

Human trials have been reported in muscular dystrophy,³⁰ neuromuscular disorders,²⁸ and McArdle's disease.²⁹ We found that CM supplementation (10 g/day \times 5 days \rightarrow 5 g/day \times 5 days) significantly increased handgrip and dorsi-flexion strength in an open trial with 81 patients with a variety of neuromuscular disorders.²⁸ Similar significant results were obtained with the 21 patients in the single-blinded arm of this study, with no effect of placebo.²⁸ Another study examined the effects of CM supplementation (adults = 10 g/day; children = 5 g/day for 8 weeks) in 36 patients with muscular dystrophy in a randomized, double-blinded, crossover study.³⁰ They reported small but significant increases in manual muscle testing, quantitative muscle testing, and improvements in task time testing, with no reported side effects (including serum CK activity).³⁰ Finally, a randomized, double-blinded, crossover study evaluated the effect of CM supplementation (0.15 g/kg/day \times 5 days; \rightarrow 0.06 g/kg/day \times 4 weeks) upon muscle function and PCr stores (MRS) in nine patients with McArdle's disease.²⁹ They found that force-time integrals (a measure of muscle power) ($p = 0.03$), surface EMG ($p = 0.03$), and PCr depletion during exercise ($p = 0.04$) were significantly higher after CM supplementation.²⁹

Although these studies are encouraging, future studies must be conducted with enough statistical power to determine whether CM supplementation enhances the quality of life and is safe and efficacious in longer-term studies. Where possible, a crossover design is most likely to have the statistical power to detect a treatment effect. One potential problem with a crossover design

comes from the fact that the muscle PCr washout time is at least 4 weeks, and we suggest that at least 5 weeks be employed in these studies. It is also critical that a proposed study have a priori knowledge of the reproducibility of the outcome measures and expected magnitude of the treatment effect. As an example, the measurement of lean mass by dual-energy X-ray absorptiometry can detect an effect of CM treatment with about 10 males in a crossover study and 20 males per group in a parallel treatment design ($\alpha = 0.05$, $\beta = 0.20$).⁷¹ Long-term crossover studies are not possible in rapidly progressive disorders (ie, SMA I, ALS) or in those where treatment could change during the course of the study (ie, acute polymyositis).

Ultimately, trials of CM treatment must address the potential efficacy in ameliorating neurological impairment (ie, weakness, atrophy), disability (ie, ability to walk up stairs, independence in personal activities of daily living), and handicap [ie, ability to function in society (working, shopping, etc)].¹¹¹ Currently, randomized double-blind trials are being conducted in the areas of neuromuscular disease (ALS, MD, hereditary motor-sensory neuropathy, and SMA I and II), neurodegenerative disorders (Huntington's chorea), and aging.

Coenzyme Q_{10}

Coenzyme Q_{10} , which is also known as ubiquinone, is an essential cofactor of the electron transport chain where it accepts electrons from Complex I and II.¹¹²⁻¹¹⁴ Coenzyme Q also serves as an important antioxidant in both mitochondria and lipid membranes.^{115,116} Coenzyme Q is a lipid-soluble compound composed of redox active quinoid moieties as well as a hydrophobic tail. The predominant form of coenzyme Q in man is coenzyme Q_{10} , containing 10 isoprenoid units in the tail, whereas the predominant form in rodents is coenzyme Q_9 , with 9 isoprenoid units in the tail. Coenzyme Q is soluble and mobile in the hydrophobic core of the phospholipid bilayer of the inner membrane of the mitochondria. It is involved in electron transport, in which it is reduced initially to the semiquinone radical, and transfers electrons one at a time to Complex III of the electron transport chain. Finally, coenzyme Q_{10} can also inhibit apoptosis in some cells.¹¹⁷

There has been considerable interest in the potential utility of coenzyme Q_{10} for treatment of mitochondrial disorders. Several reports found both clinical and biochemical improvement after treatment with coenzyme Q_{10} in patients with known mitochondrial disorders.¹¹⁸⁻¹²¹ Coenzyme Q_{10} decreases oxidative DNA damage in human lymphocytes.¹²² Coenzyme Q_{10} improved lactate/pyruvate in some patients with mitochondrial myopathies and it improved oxygen consumption in a patient with MELAS.^{123,124} In one report choreic movements and MRI abnormalities in

the subthalamic nucleus were improved after administration of coenzyme Q₁₀.¹²⁵ In contrast, one of the larger trials in mitochondrial disorders (n = 16) found that coenzyme Q₁₀ plus several antioxidant vitamins resulted in a threefold increase in serum coenzyme Q₁₀ concentration, with no measurable improvements in measurements of oxidative metabolism.¹²⁶

We demonstrated that coenzyme Q₁₀ administration increased brain mitochondrial coenzyme Q₁₀ concentrations in mature and older animals¹²⁷; however, others have found no increase in other tissues in younger animals.^{128,129} Administration of coenzyme Q₁₀ increased α -tocopherol concentrations in mitochondria, which was thought to indicate a sparing effect.¹³⁰ We also found that coenzyme Q₁₀ had cytoprotective efficacy in several animal models of neurodegenerative diseases: malonate and 3-nitropropionic acid neurotoxicity^{127,131}; MPTP toxicity in older mice, consistent with a potential therapeutic effect in Parkinson's disease¹³²; and in a transgenic animal model of amyotrophic lateral sclerosis.¹²⁷

Coenzyme Q₁₀ reduces occipital cortex lactate in Huntington's disease patients as assessed using MRI spectroscopy.⁴³ Coenzyme Q₁₀ is presently being tested in longer-term clinical trials with a larger sample size to determine whether it will have clinical efficacy in Huntington's disease and Parkinson's disease. Idebenone, which is a coenzyme Q₁₀ analogue, significantly reduces cardiac mass in patients with Friedrich's ataxia.¹³³ Idebenone treatment of a patient with Leber's hereditary optic neuropathy and paraparesis resulted in reversal of paraparesis, as well as improved brain PCr levels and reduced serum lactate.¹³⁴

Ginkgo Biloba

Ginkgo biloba is a plant extract that is a complex chemical mixture. *Ginkgo biloba* extract has neuroprotective effects against mitochondrial damage and oxidative stress. It has been shown to confer significant protective effects against the generation of lipid peroxides in brain homogenates and in rat brain synaptosomes.¹³⁵ Studies of primary cultures of cerebellar neurons showed protection against oxidative damage where *G biloba* inhibited nitric oxide production by macrophages, and free radical production as assessed by dichlorofluorescein fluorescence.¹³⁶ Finally, it protected hippocampal neurons from toxicity produced by either hydrogen peroxide or nitric oxide.¹³⁷

A beneficial effect of *G biloba* extract has been demonstrated in preventing age-induced and anoxia/reoxygenation-induced mitochondrial damage.^{138,139} An age-dependent decrease in mitochondrial function and an increase in oxidative damage to mitochondrial DNA in 24-month-old rats compared with 4-month-old rats was prevented by treatment with *G biloba*. *Ginkgo biloba* protected against MPTP-induced toxic-

ity to dopaminergic neurons.¹⁴⁰ It was also effective in models of both focal and global ischemia, which may involve both oxidative stress and mitochondrial dysfunction. We found that *G biloba* extract exerted a small but significant increase in survival in transgenic mice that model ALS.¹⁴¹ *Ginkgo biloba* extract has been reported to have a beneficial effect on cognition in individuals with AD.^{142,143}

Nicotinamide

Nicotinamide is a precursor of NADH which is a substrate for Complex I (NADH-ubiquinone oxidoreductase). It is also an inhibitor of poly-ADP-ribose polymerase, an enzyme which is activated by DNA damage and which in turn depletes both NADH and ATP. Activation of poly-ADP-ribose polymerase plays a role in neuronal injury induced by both ischemia and MPTP.^{144,145} Our studies and others showed that nicotinamide attenuated neuronal injury and ATP depletion produced by focal ischemia, malonate, and MPTP.^{131,146-148} In human studies, nicotinamide administration attenuated elevated blood lactate concentration and improved PCr/ATP in patients with MELAS.^{149,150}

Riboflavin

Riboflavin (Vitamin B₂) is the precursor of flavin mononucleotide and flavin dinucleotide, which are required for the activity of flavoenzymes involved in Complexes I and II of the electron transport chain. When given to patients with MELAS or mitochondrial myopathies, riboflavin improved clinical and biochemical abnormalities.¹⁵⁰⁻¹⁵³ It is particularly effective in patients with multiple acyl-CoA dehydrogenase deficiency, which results in a myopathy with exercise intolerance and lipid storage in muscle.¹⁵⁴⁻¹⁵⁶ In these patients riboflavin at a dose of 100 to 200 mg/day improved aerobic performance, reduced blood lactate, and normalized Complex I and II activities.

Carnitine, Acetyl-L-carnitine

Carnitine facilitates entry of long-chain fatty acids into mitochondria for subsequent β oxidation. Long-chain fatty acids, are trans-esterified to L-carnitine in a reaction catalyzed by carnitine palmitoyltransferase I (on the outer mitochondrial membrane). The acyl-carnitine is finally cleaved by carnitine palmitoyltransferase II (on the inner mitochondrial membrane) to yield an acyl-CoA moiety for subsequent β oxidation. Carnitine also facilitates the removal of short-chain and medium-chain fatty acids from the mitochondria, that accumulate during normal and abnormal metabolism. Short- and medium-chain fatty acids as acyl-CoA esters are trans-esterified to carnitine by the action of carnitine acyltransferase. The acylcarnitine esters are then transported out of mitochondria by the carnitine-

acylcarnitine translocase. This leads to regeneration of free coenzyme A under conditions in which short-chain acyl-CoA esters are produced more rapidly than they can be used.

Carnitine delayed mitochondrial depolarization in response to a variety of stressors, including oxidative damage.¹⁵⁷ Acetyl-L-carnitine increases cellular respiration, mitochondrial membrane potential, and cardioplipin levels in hepatocytes of 24-month-old rats.¹⁵⁸ These biochemical effects were paralleled by an increase in ambulatory activity of the aged rats.¹⁵⁸ Acetyl-L-carnitine increased PCr and decreased lactate in the adult and aged rat brain.¹⁵⁹ Carnitine and acetyl-L-carnitine attenuate neuronal damage produced by 3-nitropropionic acid, rotenone, and MPTP in vitro.^{160,161} Carnitine pretreatment attenuates ischemia induced reductions in ATP and PCr in mouse brain.¹⁶² After ischemia and reperfusion in rats, acetyl-L-carnitine resulted in a more rapid recovery of ATP and PCr levels, and decreased lactate.¹⁶³ In dogs subjected to ischemia/reperfusion acetyl-L-carnitine enhanced normalization of brain energy metabolites and improved neurologic outcome.¹⁶⁴ An initial study of acetyl-L-carnitine at a dose of 2 g/day appeared promising for the treatment of AD, but subsequent studies have been disappointing.¹⁶⁵

Lipoic Acid

Lipoic acid is a disulfide compound found naturally in mitochondria as the coenzyme for pyruvate dehydrogenase and α -ketoglutarate dehydrogenase. It protected against peroxynitrite induced nitration and α_1 -antiproteinase inactivation¹⁶⁶ and was neuroprotective in rodent models of both focal and global cerebral ischemia.^{167,168} In rat hepatocytes α -lipoic acid prevented an age-associated decline in ascorbic acid concentrations.¹⁶⁹ Supplementation with α -lipoic acid in old rats improved ambulatory activity, decreased oxidative damage, and improved mitochondrial function.¹⁷⁰ Dietary supplementation increased unbound lipoic acid, which can act as an antioxidant and ameliorate oxidative stress both in vitro and in vivo.¹⁷¹ In humans a dose of 600 mg/day decreased plasma indices of oxidative stress, low-density lipoprotein oxidation, and urinary isoprostanes.¹⁷¹ In one patient with chronic progressive external ophthalmoplegia and mitochondrial DNA deletions, the administration of 600 mg of lipoic acid daily for 1 month resulted in a 55% increase in brain PCr and a 72% increase in the phosphorylation potential.¹⁷²

Lipoic acid has been shown to enhance microcirculation in diabetic patients, which was thought to occur at the level of the vasa nervorum.¹⁷³ Another study found that lipoic acid reduced lipid peroxides (an indicator of oxidative stress) in ten patients with diabetic neuropathy.¹⁷⁴ A recent randomized, double-blind trial

in a total of 65 patients with diabetes found beneficial effects of lipoic acid (total of 45 in treatment group) on sural nerve amplitude and sural and tibial nerve conduction velocity.¹⁷⁵ Finally, a short-term (3 week) study in Type II diabetes found a reduction in the Total Symptom score ($p = 0.021$) and the Neuropathy Disability score ($p = 0.025$) in patients randomized to lipoic acid ($n = 12$) compared with placebo ($n = 12$).¹⁷⁶ An important issue with respect to treatment recommendations was the good tolerability of lipoic acid in the diabetic patient trials mentioned above.

Dichloroacetate

Dichloroacetate has been used as a treatment for lactic acidosis by stimulating pyruvate dehydrogenase activity through two mechanisms. First, it stimulates the activity of pyruvate dehydrogenase by inhibiting the kinase that reversibly phosphorylates and inactivates the complex. It also decreases the degradation of the E1 α -subunit of pyruvate dehydrogenase.¹⁷⁷ Pyruvate dehydrogenase, which is located on the inner mitochondrial membrane, stimulates the conversion of pyruvate to acetyl-CoA and CO₂, thus determining whether glucose is metabolized oxidatively or is converted into lactate.

In studies of ischemia and reperfusion, dichloroacetate facilitates the recovery of PCr and ATP and reduces lactate.^{178,179} After forebrain ischemia, dichloroacetate protected against neuronal damage in the CA1 region of the hippocampus and in the dorsolateral striatum in rats.¹⁸⁰ Dichloroacetate also improved energy recovery of ischemic rat hearts.¹⁸¹ We found that dichloroacetate improved motor performance and survival in a transgenic mouse model of Huntington's disease (Andreassen and Beal, unpublished).

Dichloroacetate at a dose of 25 to 50 mg/day resulted in lower lactate concentrations and clinical improvements in some patients with congenital lactic acidosis.¹⁸² Studies using proton magnetic resonance spectroscopy to monitor lactate showed improvements in patients with pyruvate dehydrogenase deficiency, MELAS, Leigh syndrome, and mitochondrial encephalomyopathies.¹⁸³⁻¹⁸⁷ An improvement of basal ganglia lesions on computed tomography and MRI was also noted in a patient with Leigh syndrome.¹⁸⁸ Dichloroacetate also reduced serum and cerebrospinal fluid lactate concentration, associated with clinical improvement, in three patients with MELAS syndrome.¹⁸⁹ A recent study has also found a trend toward a reduction in cerebral lactate/*N*-acetyl aspartate content 1 to 5 days after cerebral infarction in humans.¹⁹⁰ In contrast to the apparent tolerability of creatine and lipoic acid in healthy volunteers and patients (see above), a recent report on an ongoing open trial of dichloroacetate treatment in mitochondrial cytopathies found that 16% of patients developed new neuropathy symptoms

and 32% had a worsening of nerve conduction studies.¹⁹¹ A recent study with seven patients with a variety of mitochondrial cytopathies found that dichloroacetate reduced resting and exercise plasma lactate concentrations, yet there were no effects on muscle oxidative capacity, as determined by ³¹P-MRS.¹⁹² There is a blinded crossover study with low- and higher-dose dichloroacetate ongoing with mitochondrial disorders that should clarify issues of potential efficacy as well as side effects.¹⁹¹

Conclusions

Substantial evidence implicates impaired bioenergetics in the pathogenesis of neurodegenerative and neuromuscular disease.¹⁹³ If this is the case, agents that improve brain bioenergetics may have therapeutic utility. A substantial body of evidence suggests that creatine can exert neuroprotective effects in animal models, and initial clinical studies in humans are promising. Coenzyme Q₁₀ also appears beneficial in animal studies and is currently being tested in clinical trials in both Huntington's and Parkinson's disease. Lipoic acid appears to be promising in the treatment of diabetic neuropathy. A long-term randomized trial should clarify whether dichloroacetate has a role in the treatment of mitochondrial disorders. There is preclinical and limited clinical evidence for efficacy of other agents. Further controlled clinical trials will be needed to establish efficacy and safety of these agents in the prevention and treatment of neurological diseases. It is possible that some of the agents reviewed here could be useful in combinations that target several pathways in neurological dysfunction (eg, creatine and coenzyme Q₁₀ for reduced energy charge, combined with lipoic acid for oxidative stress).

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EXPERIMENTAL MODELS OF PARKINSON'S DISEASE

M. Flint Beal

Research into the pathogenesis of Parkinson's disease has been rapidly advanced by the development of animal models. Initial models were developed by using toxins that specifically targeted dopamine neurons, the most successful of which used 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a toxin that causes parkinsonism in man. More recently, the identification of α -synuclein mutations as a rare cause of Parkinson's disease has led to the development of α -synuclein transgenic mice and *Drosophila*. Here, I discuss the merits and limitations of these different animal models in our attempts to understand the physiology of Parkinson's disease and to develop new therapies.

BRADYKINESIA

Slowing of and difficulty in initiating movement that is characteristic of Parkinson's disease.

Parkinson's disease (PD) is the second most common neurodegenerative disease, affecting 1% of the population above the age of 65; it affects over one million people and age is the most important risk factor. The cardinal clinical manifestations of PD include BRADYKINESIA, rest tremor, rigidity, gait abnormalities and postural instability^{1,2}. The characteristic pathology is degeneration of dopamine neurons in the substantia nigra pars compacta and the presence of intracytoplasmic inclusions known as Lewy bodies (FIG. 1). Lewy bodies are small (5–25 μ m) spherical inclusions that consist of a dense granular core surrounded by a halo of radiating filaments. They are consistently immunostained with antibodies to α -synuclein and ubiquitin. There is a loss of dopamine and its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetate (DOPAC), and the dopamine transporter in the striatum, as well as in the substantia nigra pars compacta. Less extensive neuronal death occurs in other brainstem catecholamine and serotonin nuclei, hypothalamic neurons, small cortical neurons and in the nucleus basalis of Meynert, the main provider of cortical acetylcholine.

Brain imaging is rarely useful in the diagnosis of PD, but positron-emission tomography has confirmed a loss of dopamine terminals in patients. Several other disorders produce parkinsonism and misdiagnosis occurs in about 24% of cases¹. The most

reliable clinical features to distinguish PD from parkinsonism due to other causes are asymmetry of symptoms and signs, the presence of resting tremor, and a good response to levodopa, a biosynthetic precursor of dopamine.

The specific aetiology of PD is unknown and this has made it difficult to develop ideal methods for studying the disease. There is evidence for a role of both environmental and genetic factors^{3–5}. Post-mortem studies have provided consistent evidence for both oxidative damage and a decrease in activity of complex I of the mitochondrial electron transport chain in the substantia nigra. The discovery of mutations that cause familial PD has spurred research and led to the generation of new animal models. Rare missense mutations in the α -synuclein protein, Ala53 \rightarrow Thr and Ala30 \rightarrow Pro, cause autosomal dominant PD in a large Italian/American/Greek family and a small German pedigree, respectively⁶. In addition, autosomal-recessive juvenile-onset PD is caused by mutations in the gene parkin⁷. Here, I discuss the merits of the available animal models of PD, both for disease pathophysiology and for testing experimental therapeutics.

Desirable characteristics for a model of PD

For direct relevance to human PD, an ideal animal model should have the following characteristics (TABLE 1): first, a normal complement of dopamine

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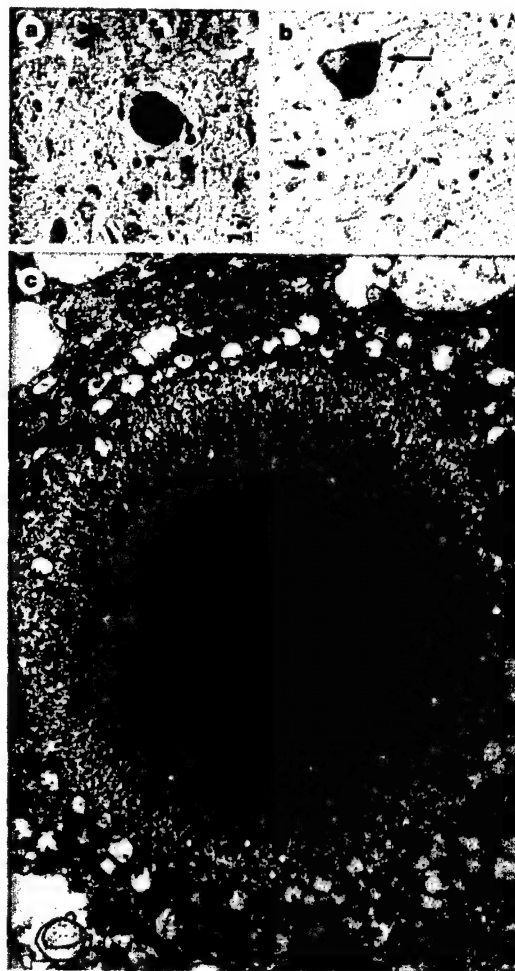


Figure 1 | Lewy bodies are a characteristic feature of Parkinson's disease. Lewy bodies are small spherical inclusions that **a** | can be stained with haematoxylin/eosin, and **b** | contain the protein α -synuclein, which in this specimen was detected with a specific antibody. **c** | Lewy bodies consist of a dense granular core that is surrounded by a halo of radiating filaments that can be seen in this electron micrograph. (Panels **a** and **b** © Macmillan Magazines Ltd; panel **c** is adapted with permission from REF. 105 © (1996) American Association of Neuropathologists.)

neurons at birth with selective and gradual loss of dopamine neurons commencing in adulthood. The losses should exceed 50% and be readily detectable using biochemistry and neuropathology. Second, the model should have easily detectable motor deficits, including the cardinal symptoms of PD, which are bradykinesia, rigidity and resting tremor. Previous experience with pharmacological depletion of dopamine (reserpine) or dopamine-receptor antagonists, however, suggests that mice do not show rigidity or resting tremor. Third, the model should show the development of characteristic Lewy bodies. Fourth, if the model is genetic, it should be based on a single mutation to allow robust propagation of the mutation, as well as crossing with enhancer or suppressor strains. Last, it should have a relatively short disease course of a few months, allowing rapid and less costly screening of therapeutic agents.

AMPHETAMINE

Molecule that inhibits dopamine uptake and increases the level of this transmitter in the cleft. Its intake by normal subjects induces a psychotic state.

POLYMORPHISM

The simultaneous existence in the same population of two or more genotypes in frequencies that cannot be explained by recurrent mutations.

Current animal models of PD

6-hydroxydopamine. This was the first agent used to model PD. After injection of 6-hydroxydopamine into the substantia nigra, this drug selectively accumulates in dopamine neurons, and it then kills these neurons owing to toxicity that is thought to involve the generation of free radicals (FIG. 2). 6-hydroxydopamine is an effective toxin in rats, mice, cats and primates that has been predominantly used to produce unilateral lesions. In rats, the extent of dopamine depletion can then be assessed by examining rotatory behaviour in response to AMPHETAMINE and apomorphine⁸. Other behavioural deficits, such as paw reach, stepping and sensory neglect tasks, also occur. It has therefore proved to be useful for pharmacological screening of agents that have effects on dopamine and its receptors. A more chronic model was reported by Sauer and Oertel⁹. They injected 6-hydroxydopamine into the striatum in rats and reported a progressive loss of substantia nigra neurons to 96% of control of the contralateral side at 1 week, 59% at 2 weeks, 35% at 4 weeks, 23% at 8 weeks and 15% at 16 weeks. This seems to be a consequence of loss of axon terminals followed by retrograde degeneration. 6-hydroxydopamine lesions do not result in Lewy bodies in the substantia nigra, and can produce nonspecific damage to other neurons. A major advantage of this model, however, is a quantifiable motor deficit (rotation).

Rotenone. The possibility that pesticides and other environmental toxins are involved in the pathogenesis of PD has been suggested by several epidemiological studies^{3,4}. Patients with certain glutathione transferase POLYMORPHISMS and exposure to pesticides seem to have an increased incidence of PD¹⁰. Atypical PD is associated with consumption of fruits and herbal tea containing insecticides in the French West Indies¹¹. Rotenone is a naturally occurring compound derived from the roots of certain plant species, which has been used as an insecticide in vegetable gardens, and to kill or sample fish populations in lakes or reservoirs. Rotenone is known to be a high-affinity specific inhibitor of complex I, one of the five enzyme complexes of the inner mitochondrial membrane that are involved in oxidative phosphorylation. As mentioned above, deficits in complex I have been observed in the substantia nigra of people with PD (FIG. 2).

Betarbet and colleagues produced a model of PD by infusing rats intravenously with rotenone by jugular vein cannulas attached to subcutaneous osmotic minipumps¹². This mode of administration was necessary owing to the extreme hydrophobicity and insolubility of rotenone in aqueous solvents. It was shown that Lewis rats were less susceptible than Sprague-Dawley rats, but they produced much more reliable lesions, consistent with observations made using the mitochondrial toxin 3-nitropropionic acid¹³. The rats developed a progressive degeneration of nigrostriatal neurons with a loss of tyrosine hydroxylase, dopamine transporter and vesicular monoamine transporter immunoreactivity. Furthermore, the nigral neurons showed cytoplasmic

Table 1 | Characteristics of animal models of Parkinson's disease

Model	Gradual loss of dopamine neurons in adulthood	Easily detectable motor deficits	Development of Lewy bodies	Based on a single mutation	Short timecourse
6-hydroxydopamine	No	Yes (quantifiable rotation deficit)	No	N.A.	Yes
Rotenone	Yes, but variable individual susceptibility	Yes	Yes	N.A.	Yes
Acute MPTP*	No	Yes	No	N.A.	Yes
<i>Drosophila</i> α -synuclein overexpression	Yes	Yes	Yes	Yes	Yes
Mouse α -synuclein overexpression	Yes, but not in the substantia nigra	Yes	Nuclear as well as cytoplasmic inclusions	N.A.	No (1 year)

*Chronic MPTP administration produces slow evolution of a parkinsonian syndrome and might produce Lewy bodies. (MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; N.A., not applicable.)

inclusions that were reminiscent of Lewy bodies. The inclusion bodies stained with antibodies to ubiquitin and α -synuclein, and electron microscopy showed a dense core surrounded by fibrillar elements similar to Lewy bodies. The rats showed bradykinesia, postural

instability, unsteady gait and some evidence of tremor, and the deficits improved after treatment with the dopamine receptor agonist apomorphine.

These findings are remarkable because they show that an inhibitor of complex I of the electron transport chain, which acts uniformly throughout the brain, can produce selective degeneration of nigrostriatal neurons. They therefore indicate that substantia nigra neurons are particularly vulnerable to complex I inhibitors. This is consistent with findings of decreased complex I activity in PD post-mortem tissue and platelets¹⁴. This effect might be mediated by oxidative damage, as the brain rotenone concentration was enough to only partially inhibit complex I, and not high enough to significantly impair respiration. This model meets most of the criteria needed for an excellent animal model of PD. However, it suffers from much variability in the susceptibility of individual rats to the toxin; only 12 out of 25 rats treated with standardized doses of rotenone developed lesions. Such a degree of variability unfortunately precludes the usefulness of the model for testing neuroprotective agents. This model could, however, be valuable in examining cell replacement strategies such as transplantation of fetal nigral neurons or stem cells, and it might lead to novel insights into the pathophysiology of the death of substantia nigra neurons in PD.

***Drosophila* model of PD.** As mentioned above, mutations in α -synuclein, a protein found in Lewy bodies, are related to PD in some pedigrees. A novel model of PD was produced by Feany and Bender by expressing mutant and normal forms of α -synuclein in *Drosophila*¹⁵. These authors showed that flies overexpressing both wild-type and mutant α -synuclein showed an age-dependent loss of dorsomedial neurons that stained for tyrosine hydroxylase. Other subsets of dopamine neurons, however, showed no abnormalities, consistent with the differential vulnerability of dopamine neurons in human PD, and cortical neurons and serotonin neurons were spared. These authors also observed α -synuclein-stained inclusions in neurons, which resembled Lewy bodies by light microscopy and electron microscopy. The flies developed locomotor dysfunction with age, but it was not

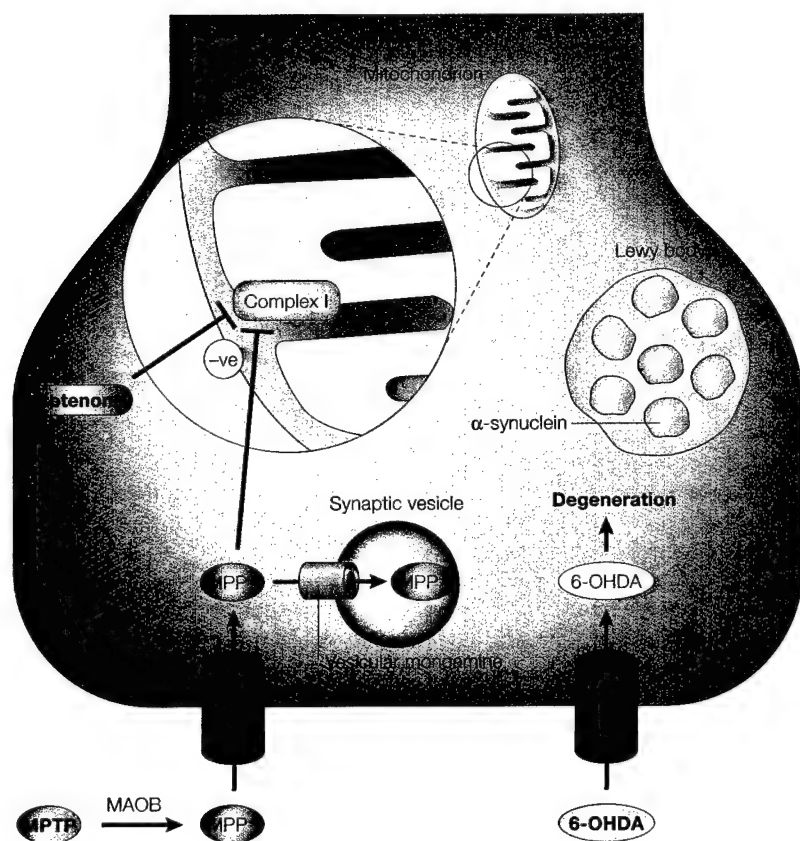


Figure 2 | Pathogenesis of neuronal dysfunction produced by neurotoxins that affect dopamine neurons. The mechanisms by which neurotoxins kill dopamine neurons involve mitochondrial dysfunction and oxidative damage. 6-hydroxydopamine (6-OHDA) is taken up by the dopamine transporter and it then generates free radicals. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is converted by monoamine oxidase B (MAOB) to 1-methyl-4-phenylpyridinium (MPP⁺). MPP⁺ is taken up by the dopamine transporter and can then be accumulated by mitochondria, leading to complex I inhibition and the generation of free radicals, or by the vesicular monoamine transporter, thus reducing toxicity. Rotenone is a direct inhibitor of complex I, which also leads to free-radical generation. MPTP and rotenone treatment increase the expression of α -synuclein and, in the latter case, this leads to the formation of Lewy bodies.

determined if these deficits could be reversed by the use of dopamine receptor agonists. There was also retinal degeneration, which is not a typical feature of PD, but this could be a unique feature of the *Drosophila* model. Also, it is not clear as yet that the motor deficits observed are due to dysfunction of dopamine neurons. Furthermore, there was no difference in the toxicity of wild-type and mutant α -synuclein. Nevertheless, the well-characterized genetics of *Drosophila* will allow the rapid characterization of enhancer and suppressor mutations — an important advantage.

Mouse α -synuclein models. Mice that lack the α -synuclein gene are viable, fertile and show normal brain structure, as well as no deficits in dopamine cell bodies, fibres and terminals¹⁶. They show no evidence of a parkinsonian phenotype, suggesting that a loss of function of α -synuclein is unlikely to cause PD, and that mutations in α -synuclein are therefore more likely to cause PD by a gain of function. The α -synuclein mice, however, show a mild reduction in striatal dopamine (18%), an attenuation of dopamine-dependent locomotor response to amphetamine and increased dopamine release following paired stimuli, suggesting that α -synuclein is a presynaptic activity-dependent regulator of dopamine-mediated neurotransmission¹⁶.

Mice overexpressing wild-type α -synuclein, using the platelet-derived growth factor (PDGF) promoter, develop a progressive accumulation of α -synuclein- and ubiquitin-immunoreactive inclusions in the neocortex, hippocampus and substantia nigra¹⁷. However, there were both cytoplasmic and nuclear inclusions, and the latter are not a typical feature of PD. Electron microscopy showed that the inclusions were composed of fine granular material, but the fibrillar aggregates that are characteristic of Lewy bodies were not reported. There was no loss of dopamine neurons within the substantia nigra, but there was a loss of dopamine terminals in the line with the highest expression level as assessed by immunocytochemistry. There was also a decrease in tyrosine hydroxylase protein levels and activity, although dopamine levels were not measured. The mice showed impairment on ROTAROD TESTING at one year, but it was not determined if these deficits were reversible with dopamine receptor agonists. This model therefore lacks several of the characteristic features of PD.

In transgenic mice with overexpressed wild-type or mutant α -synuclein under the control of a Thy-1 promoter, rotarod testing revealed a progressive loss of motor function; however, it was not determined if this was responsive to dopamine receptor agonists¹⁸. There was increased α -synuclein staining in ~80% of motor neurons and some also showed Lewy-body-like pathology. Neuronal and dendritic α -synuclein staining was increased in other telencephalic and brainstem neurons, although not in substantia nigra neurons in which the Thy-1 promoter is not expressed. So, this model produces some aspects of the pathology of dementia with Lewy bodies, but it does not faithfully replicate PD.

MPTP model. The best-characterized model of PD has been developed by using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)¹⁹. The discovery of MPTP occurred in 1982 when a group of drug addicts in California developed subacute onset of severe parkinsonism. Investigation revealed that the syndrome was caused by self-administration of a synthetic heroin analogue that had been contaminated by a byproduct — MPTP — during manufacture. MPTP administration was subsequently shown to model PD in both mice and primates. MPTP is highly lipophilic and it readily crosses the blood-brain barrier. It is then converted into its active metabolite, 1-methyl-4-phenylpyridinium (MPP⁺) by monoamine oxidase B, an enzyme involved in catecholamine degradation (FIG. 2). MPP⁺ is taken up by the plasma-membrane dopamine transporter, for which it has a high affinity; mice that lack this transporter are protected from MPTP toxicity²⁰. MPP⁺ can be taken up by vesicular monoamine transporters (VMAT), and mice with 50% depletion of VMAT show increased vulnerability to MPTP²¹. Intracellular MPP⁺ is taken up and concentrated in mitochondria, where it inhibits complex I of the electron transport chain²². This reduces ATP generation, but a more important effect might be increased free-radical production²³. Indeed, MPTP toxicity is significantly reduced in transgenic mice overexpressing the free-radical scavenging enzymes Cu/Zn superoxide dismutase and Mn superoxide dismutase^{24,25}. Conversely MPTP toxicity is exacerbated in mice with deficiencies of glutathione peroxidase, Cu/Zn superoxide dismutase or Mn superoxide dismutase^{26–28}. The critical oxidant, however, might be peroxynitrite, which is formed by the chemical reaction of an NO radical with an O₂ radical. 3-nitrotyrosine, a marker for peroxynitrite-induced damage, is increased with MPTP toxicity^{29,30} and in PD brains³¹. Knockout of neuronal nitric oxide synthase in mice or inhibitors of neuronal nitric oxide synthase markedly attenuate MPTP neurotoxicity^{27,30,32–34}. Mice that lack the inducible form of nitric oxide synthase are also resistant to the toxin. However, the protection was confined to cell bodies and did not occur in dopamine terminals^{35,36}. Oxidative damage to DNA can activate poly-ADP-ribose polymerase, an enzyme that is involved in DNA repair. Activation of poly-ADP-ribose polymerase further depletes cellular ATP and NAD. Knockout of poly-ADP-ribose polymerase in mice or the use of inhibitors against this enzyme significantly attenuate MPTP neurotoxicity^{37–39}.

MPTP toxicity in primates replicates all the clinical signs of PD, including tremor, rigidity, akinesia and postural instability^{19,40}, and it has been useful for studying the striatal circuitry involved in PD pathophysiology (BOX 1). Primates treated with MPTP show an excellent response to L-DOPA (3,4-dihydroxyphenylalanine) and dopamine receptor agonists. As in PD, the substantia nigra pars compacta neurons are particularly vulnerable, whereas neurons in the ventral tegmental area, a dopamine nucleus that largely projects to the cortex, are more resistant. The toxin also causes a loss of locus coeruleus neurons, cells that are also lost in PD⁴¹. In monkeys injected chronically with low doses of MPTP,

ROTAROD TEST

Motor test that probes the ability of rodents to keep their balance on a cylinder that rotates continuously at a slow speed, commonly 5–6 revolutions per minute.

Box 1 | **Modelling the physiology of Parkinson's disease**

The MPTP model of Parkinson's disease (PD) in primates has been invaluable in studying striatal circuitry involved in the pathophysiology of PD. The basal ganglia is organized into larger segregated circuits involving the thalamus and cerebral cortex. Electrophysiological studies in the MPTP model have shown that there is abnormal firing of neurons in output pathways. After treatment with MPTP, there is reduced tonic activity of neurons in the external globus pallidus, whereas it is strongly increased in the subthalamic nucleus, internal globus pallidus, and substantia nigra pars reticulata^{49,100–102}. This in turn might lead to increased inhibition of thalamocortical neurons. In support of this model, lesions of the subthalamic nucleus, the internal globus pallidus or the substantia nigra reticulata ameliorate the motor signs of PD in MPTP-treated primates^{48,49}. These findings led to a resurgence of interest in surgical procedures for treating PD. It has subsequently been shown that stereotactic lesions of the internal globus pallidus are effective for reversing the signs of PD, and for reducing the involuntary movements and the fluctuating response to levodopa that occurs in advanced PD¹⁰³. Deep brain stimulation of the subthalamic nucleus, which produces reversible inactivation, is also highly effective in relieving the symptoms of PD¹⁰⁴.

the loss of dopamine terminals is greater in the putamen, which is similar to PD in humans. In aged MPTP-treated primates, there were neuronal inclusions that partially resembled Lewy bodies⁴², but typical electron-microscopic features were not observed⁴³. The main difficulty with MPTP toxicity as a model of PD, however, is that it is an acute or subacute process, whereas PD is a slowly progressive illness that evolves over years. With this in mind, chronic administration of MPTP in primates using several dosing regimes has been shown to produce slow evolution of a parkinsonian syndrome, with uneven striatal dopamine fibre loss and more selective depletion of substantia nigra dopamine neurons^{41,44–46}. Another model that has been valuable is to produce hemiparkinsonism by unilateral internal carotid artery infusion of MPTP⁴⁷. This model has been useful for studying novel antiparkinsonian therapies as well as surgical interventions^{48,49}.

How does MPTP administration lead to neuronal death? There is some evidence that apoptosis could be involved. In mice, a subacute dosing regime of MPTP daily over five days was shown to induce cell death by apoptosis, as assessed by terminal deoxynucleotidyl transferase labelling (TUNEL), and acridine orange staining to visualize clumped chromatin⁵⁰. Several studies have supported an involvement of apoptosis in PD with increased caspase-3 and Bax immunoreactivity in melanin-containing neurons, which are characteristic of the substantia nigra⁵¹. There is also evidence for increased TUNEL staining and chromatin condensation in PD nigral neurons⁵²; however, this has not been found by others⁵³. To further investigate the role of apoptosis in MPTP toxicity, we studied its effects in mice that overexpress Bcl-2 (REF. 54). We found significant neuroprotection, but surprisingly it was greater for an acute rather than a chronic administration over five days (which previously was shown to produce apoptotic cell death). Others obtained similar results⁵⁵. We also found marked protection against MPTP neurotoxicity in transgenic mice with a dominant-negative inhibition of caspase-1 (REF. 56). Mice deficient in the pro-apoptotic protein Bax are also resistant to MPTP toxicity⁵⁷.

Adenovirus administration of a protein caspase inhibitor — X-chromosome-linked inhibitor of apoptosis (XIAP) — protects dopamine cell bodies but does not protect terminals, whereas a combination of the growth factor GDNF (glial-cell-derived neurotrophic factor) with XIAP protects both cell bodies and terminals⁵⁸. Mice with a knockout of p53, which is a growth-control gene involved in programmed cell death, were resistant to MPTP-induced death of dopamine neurons⁵⁹. MPTP activates c-Jun N-terminal kinase (JNK), which acts as a mediator of MPTP-induced apoptotic cell death, as inhibitors of JNK are neuroprotective^{60,61}. The anti-apoptotic agent CGP3466 produced partial protection against MPTP-induced loss of nigral neurons⁶².

In addition to the possible role of apoptosis, a role for inflammation in the pathogenesis of MPTP toxicity is supported by both human and animal studies. Neuropathological studies of three patients exposed to MPTP 3–16 years previously showed clustering of microglia around neurons and active ongoing cell loss⁶³. After MPTP administration in mice, there is evidence for both activated microglia and lymphocytes⁶⁴. Inhibition of cyclooxygenase isoenzymes 1 and 2 with acetylsalicylic acid, or of cyclooxygenase-2 selectively with meloxicam, produces significant protection⁶⁵. This suggests that cyclooxygenase-2 might be a significant source of superoxide radicals in the cell. Mice that lack cytoplasmic phospholipase A2, a mutation that blocks the production of substrate for cyclooxygenase-2, are markedly protected against MPTP toxicity⁶⁶. Other studies showed partial neuroprotection with sodium salicylate. However, this agent can also act as a free-radical scavenger^{67,68}.

In addition to free-radical scavengers, the availability of the MPTP model has made it possible to examine other neuroprotective strategies. Glutamate receptor antagonists have produced partial protection^{69–71}. An adenosine A1 receptor agonist showed dose-dependent protection⁷². Riluzole has produced some protection in both mice and primates^{73,74}. The dopamine D2 receptor agonists bromocriptine and pramipexole have antioxidant activity and they attenuate MPTP-induced dopamine depletion^{75,76}. Ebbsen, which is a glutathione peroxidase analogue, prevents both neuronal loss and clinical symptoms produced by MPTP in primates⁷⁷. Several free-radical spin traps also exert neuroprotective effects^{74,78}. Both erythropoietin and 17 β -oestradiol, which scavenges free radicals, exert neuroprotective effects against MPTP in mice^{79,80}.

Another neuroprotective strategy that has been tested using the MPTP model is the administration of growth factors. GDNF shows both protective and restorative effects after MPTP administration in mice and primates^{81–83}. A recent study examined the effects of LENTIVIRAL vector delivery of GDNF in primates treated with MPTP⁸⁴. In the MPTP-treated monkeys, lenti-GDNF reversed functional deficits, and completely prevented nigrostriatal degeneration when administered one week after the MPTP. The monkeys showed improvement in arm reaching, normalized fluoro-DOPA uptake on PET

LENTIVIRUSES

A group of retroviruses that include HIV. Virus derivatives that are engineered to be replication-defective can be used as expression vectors. Lentiviral vectors have advantages over retroviral vectors because of their ability to infect non-dividing human cells, particularly neurons.

scans and no significant loss of tyrosine-hydroxylase-positive neurons in the substantia nigra. Another promising approach for both neuroprotective and restorative effects are the neuroimmunophilin compounds. These compounds stimulate neurite outgrowth, and are related to FK506 but do not possess its immunosuppressive effects. Two of these compounds were shown to potently inhibit MPTP toxicity in mice, and to cause regenerative sprouting from spared nigrostriatal dopamine neurons^{85,86}.

As mentioned above, there is substantial evidence that a mitochondrial deficiency of complex I activity might be involved in the pathogenesis of PD¹⁴. The evidence comes from studies in post-mortem tissue of PD patients, as well as studies in platelets and cybrid cell lines (reviewed in REF. 14). Furthermore, both rotenone and MPTP neurotoxicity are linked to inhibition of complex I activity. This raises the possibility that agents that can modulate mitochondrial bioenergetics might exert neuroprotective effects, and this idea has been tested using the MPTP model. Acetyl-L-carnitine is an agent involved in fatty acid transport in mitochondria, and has been shown to protect against MPTP-induced parkinsonism in primates⁸⁷. Coenzyme Q₁₀ is an essential cofactor of electron transport between complex I and complex III of the electron transport chain, and it is a potent antioxidant in mitochondria. We found reduced coenzyme Q₁₀ levels in platelets of PD patients and that coenzyme Q₁₀ protected against MPTP-induced damage in aged mice^{88,89}. As a consequence, coenzyme Q₁₀ is currently being tested in PD patients. We also investigated whether creatine supplementation, which leads to increased brain phosphocreatine levels and might inhibit the mitochondrial permeability transition, can exert neuroprotective effects against MPTP neurotoxicity. We found that creatine administration exerts dose-dependent neuroprotective effects that are optimal with a concentration of 2% in the diet⁷⁸.

The most striking difference between the MPTP model and PD is the lack of Lewy-body formation. This, however, most probably relates to the fact that most paradigms of MPTP administration use acute or subacute dosing. Chronic administration of rotenone produces inclusions that closely resemble Lewy bodies, and we found that administration of MPTP to baboons over seven days produced α -synuclein aggregates in the substantia nigra⁹⁰. It is therefore possible that more chronic administration of MPTP could produce Lewy bodies.

Lessons from animal models of PD

So far, there are three neurotoxic animal models of PD, with 6-hydroxydopamine, rotenone and MPTP, respectively. There are transgenic mouse and *Drosophila* models of α -synuclein mutations and wild-type overexpression. This raises several important issues about the relevance of these animal models to PD itself. There is substantial evidence for involvement of both environmental and genetic factors in PD. It is likely that PD will prove to be heterogeneous in its aetiology, with genetic factors predominating in some cases, and environmen-

tal factors in others. It is therefore as yet unclear whether toxin or genetic models will prove to be most useful. Nevertheless, genetic models are desirable, as transgenic models of amyotrophic lateral sclerosis and Huntington's disease produce a delayed onset and more chronic neurodegenerative process that mimics human illness better than the neurotoxin models^{91,92}. So far, however, the transgenic mouse models of α -synuclein have not successfully replicated the cardinal features of PD, including a selective depletion of dopamine neurons. It is possible that with ageing or exposure to oxidative stress, these models might result in more selective damage to dopamine neurons. Oxidative stress can promote α -synuclein aggregation⁹³. The *Drosophila* model might be more successful in that there is a selective degeneration of dopamine neurons. It also shows progression, age-dependence and the formation of fibrillar α -synuclein inclusions, and has the advantage that it is readily amenable to genetic manipulations such as enhancer or suppressor mutations. Another genetic mutation that causes PD affects parkin, which is autosomal-recessive and causes the loss of activity of a ubiquitin protein ligase^{28,94}. It is possible that the creation of new transgenic mice with parkin mutations might lead to an improved model.

For the present, and on the basis of the five considerations put forward earlier (TABLE 1), the best model seems to be that produced by MPTP. It produces almost all the cardinal features of PD in primates with the exception of Lewy bodies. In mice, this model is efficient for testing neuroprotective and neurorestorative treatments. It has been shown that varying dosing regimes might favour necrotic versus apoptotic cell death. A link to α -synuclein are the findings that MPTP increases α -synuclein expression in both mice and primates^{90,95}. Furthermore, MPTP induces nitration of α -synuclein in MPTP-treated mice⁹⁶, and nitration of α -synuclein has been shown in PD⁹⁷. Interestingly, α -synuclein overexpression in a hypothalamic neuronal cell line produced inclusions, morphological abnormalities of mitochondria, mitochondrial dysfunction and increased free-radical production⁹⁸. Expression of mutant α -synuclein in NT2/D1 cells was associated with increased markers of oxidative damage and increased susceptibility to cell death induced by H₂O₂ or MPP⁺ (REF. 99). These findings therefore provide a potential link between α -synuclein and both mitochondrial dysfunction and oxidative damage, which are strongly implicated in PD pathogenesis.

Conclusions

Currently available animal models of PD have contributed greatly to our understanding of both the pathophysiology and potential neuroprotective therapeutics for PD, but as yet we do not have the optimal model. At present, MPTP neurotoxicity is the best available animal model from several standpoints, and it has been extremely valuable in testing neuroprotective and neurorestorative strategies. Nevertheless, a transgenic mouse model that accurately replicates the cardinal features of PD would be a significant advance, in that it would presumably mimic the

delayed and chronic degeneration that occurs in dopamine neurons in human PD. An ideal model for testing neuroprotective strategies should show both phenotypic and neuropathological changes within six months. If the disease phenotype occurs beyond six months, there is much greater variability, which increases the number of mice that need to be tested, and also leads to much greater expense for both housing and testing. It is highly likely that advances in genetics will lead to improved mouse models of PD in the next few years. This will undoubtedly contribute

to both an improved understanding of the pathophysiology of PD, as well as the development of novel therapeutic strategies for this devastating illness.

Links

DATABASE LINKS Parkinson's disease | α -synuclein | ubiquitin | parkin | caspase-3 | Bax | Bcl-2 | caspase-1 | XIAP | GDNF | p53 | JNK | cyclooxygenase-2 | phospholipase A2 | adenosine A1 receptor | dopamine D2 receptor | amyotrophic lateral sclerosis | Huntington's disease
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